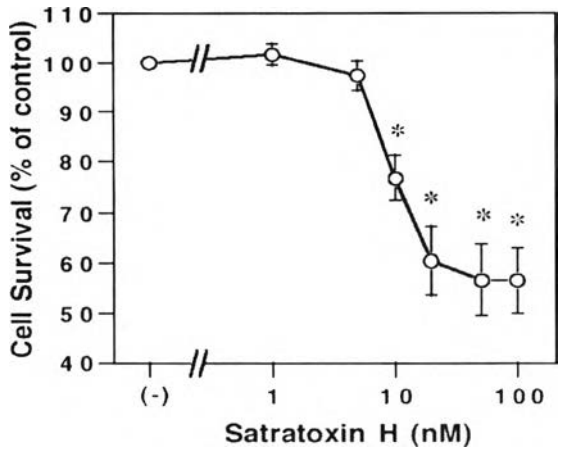
CHAPTER IV

RESULTS

1. Effect of satratoxin H on the viability of PC12 cells

To examine the satratoxin H-induced cytotoxicity, cell viability was determined in PC12 cells using MTT assay. Since active mitochondria of living cells can cleave MTT to produce formazan, the amount of formazan is directly related to the number of living cells. The doubling time of PC12 cells has been reported as 92 h (Greene and Tischler, 1976). After seeding in 96-well plate, PC12 cells were cultivated in medium for 48 h resulting in the cell population in log phase. Then PC12 cells were quiescent by incubation in serum-free medium for 24 h before treated with satratoxin H which has been demonstrated by Bello *et al.*, 2005 and Yoshizumi *et al.*, 2002. The result showed that satratoxin H reduced the viable cells 24 h after its treatment in a concentration-dependent manner with a range from 1 to 100 nM. Satratoxin H exhibited significant cytotoxicity with the IC_{50} value about 50 nM (Figure 6A). The exposure of PC12 cells to 50 nM satratoxin H resulted in a time-dependent decrease of viable cells. Cell survival was gradually declined from 6 h (74.75 ± 7.45) to 24 h (50.17 ± 6.37) as shown in Figure 6B.

(A)



(B)

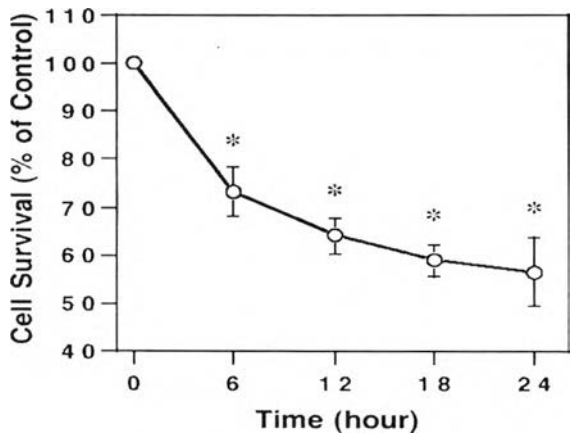


Figure 6 Satratoxin H-induced cytotoxicity of PC12 cells. (A) Concentration-dependency of satratoxin H-induced cytotoxicity in the cells incubated for 24 h. (B) Time course of satratoxin H-induced cytotoxicity. All cells were exposed to serum-free medium for 24 h before treated with satratoxin H at the concentration of 50 nM. Data were expressed as % of control cell survival measured by MTT assay. Each point represented the mean±SEM of four different experiments, each performed in triplicate. $*P<0.05$ compared to the cells without satratoxin H.

2. Satratoxin H induced apoptosis in PC12 cells

To investigate whether the reduction in mitochondrial function might lead to apoptotic cell death, morphological observation by Hoechst 33342 staining, DNA fragmentation and flow cytometric analysis were performed.

2.1 Fluorescence microscopic assay using Hoechst 33342 assay

Hoechst 33342 staining was used to evaluate whether the cell death induced by satratoxin H might be apoptosis. Hoechst 33342 stained nuclei of all cells. To demonstrate the time-course study of satratoxin H-induced apoptosis, PC12 cells were treated with satratoxin H at the concentration of IC_{50} (50 nM) in serum-free medium for 12, 24, 36, and 48 h. Satratoxin H incubated in medium with serum was performed as a control medium. The control cells incubated in medium with serum exhibited the normal shape of living cells (Figure 7A). Very few dead cells were seen in the untreated controls. In contrast, most of cells treated with satratoxin H for 24 h exhibited the typical morphological changes of apoptosis, namely nuclear condensation, fragmented chromatin accumulation to the inside of nucleolus membrane with a shape like crescent moon or ring, nuclear cleavage and apoptotic bodies (Figure 7B). Incubation in serum-free medium showed that PC12 cells exhibited in time-dependency up to 24 h and the significantly increased number of cells stained with Hoechst 33342 after treated with satratoxin H for 24 h ($P < 0.05$) after that the apoptosis slightly decreased until 48-h incubation (Figure 7C). In contrast, the percentage of apoptotic cells in satratoxin H-treated PC12 cells incubated in medium with serum gradually increased, and the highest percentage of apoptotic cells accumulated to 17.09 ± 1.22% following treatment for 48 h. However, some cells were also stained with trypan blue (data not shown) suggesting that satratoxin H could cause necrosis as well. Since Hoechst 33342 labels early and late apoptosis, it could be concluded that apoptosis in satratoxin H-treated PC12 cells appeared after 24-h incubation both in medium with serum and in serum-free medium.

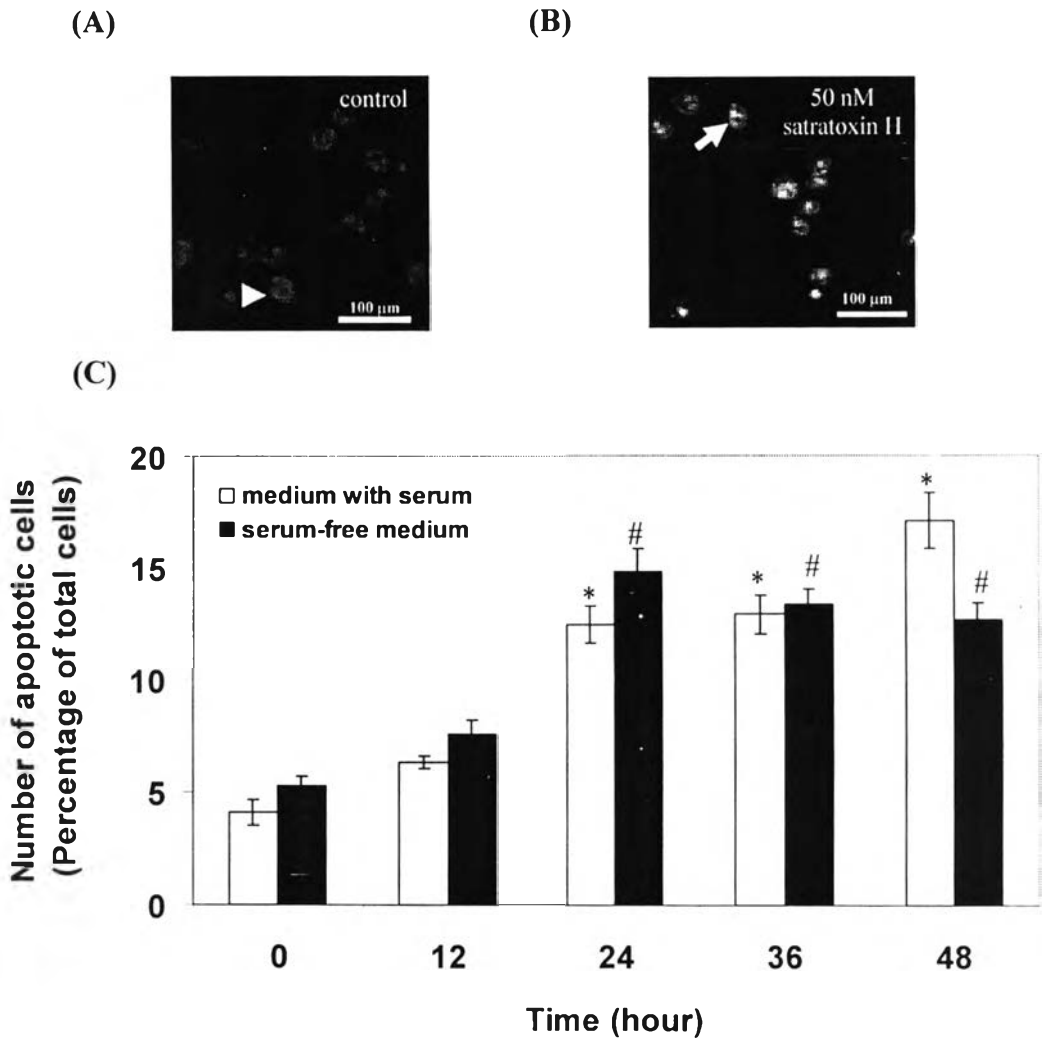


Figure 7 Morphological analysis of nuclear chromatin in PC12 cells stained with Hoechst 33342 using fluorescence microscope. In control, PC12 cells were cultivated in serum-free medium for 24 h (A), viable cells showed bright round nuclei with Hoechst 33342 staining. Apoptotic cells were hardly observed in control medium (arrow head). Satratoxin H (50 nM)-treated cells in serum-free medium for 24 h (B) displayed chromatin condensation (arrow) and nuclear fragmentation. The bar indicated time points (12, 24, 36, and 48 h) of satratoxin H-induced apoptosis cultivated in serum-free medium or medium with serum (C). Data were expressed as % of total cells. Each point represented the mean ± SEM of four different experiments, each performed in triplicate. $^{*}P<0.01$ compared to control in medium with serum, $^{\#}P<0.01$ compared to control in serum-free medium. Scale bar = 100 μm.

2.2 DNA fragmentation analysis using agarose gel electrophoresis

Since Hoechst 33342 staining represented the induction of early and late apoptotic cell death, satratoxin H-treated PC12 cells was further determined for DNA fragmentation, represented late apoptosis. Apoptotic cells often produce a unique ladder composed of nucleotide fragments at an interval of 180-200 base pairs, which can be visualized by agarose gel electrophoresis. The internucleosomal DNA digested by endogenous nucleases yield a characteristic laddering pattern is one of the hallmarks of apoptotic cell death. PC12 cells were harvested at various times (24, 48, and 72 hours) after satratoxin H was incubated in both complete medium and in serum-free medium as a control medium. Total DNA was purified for the agarose gel electrophoresis assay. The 72-h incubation of 50 nM satratoxin H in medium containing serum showed DNA ladder (*lane 4*), but 24- and 48-h incubation did not (*lane 2, 3*). PC12 cells incubated with satratoxin H in medium without serum clearly exhibited DNA ladder after 24-h incubation (*lane 6*), which was after 48-h incubation (*lane 7*), and hardly seen after 72-h incubation *(lane 8*) of satratoxin H. This result confirmed the result from Hoechst 33342 staining which showed that the percentage of apoptotic cell death significantly increased ($P<0.01$) after 24-h incubation of satratoxin H both in serum with medium and serum-free medium (Figure 7C). The decrement of DNA ladder after 48- and 72-h incubation in serum-free medium may be from apoptotic cell death changed into necrotic cell death, respectively. However, 72-h cultivation of PC12 cells in medium with serum (*lane 1*) did not show DNA ladder, but in serum-free medium (*lane 5*) showed intrinsic pattern of DNA ladder (Figure 8). Data implied the efficient induction of apoptosis in satratoxin H-treated PC12 cells, which was clearly seen after 24-h incubation of satratoxin H in serum-free medium and after 72-h incubation of satratoxin H in medium with serum.

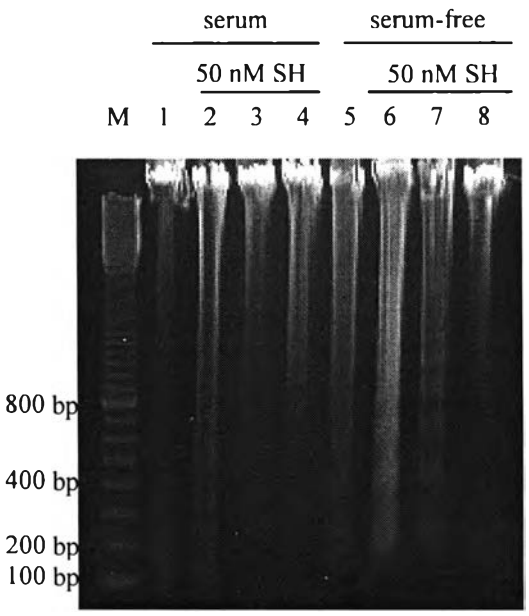
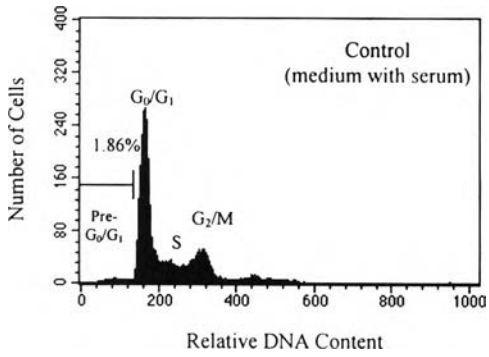


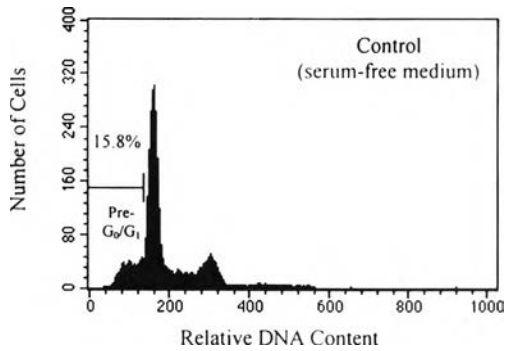
Figure 8 Induction of DNA fragment laddering by satratoxin H (50 nM). DNA was extracted as mentioned in "method" and electrophoresis of DNA (50 μg) on a 1.5% agarose gel. Lanes refer to the following: *lane M*, 100 base pair DNA ladder; *lane 1*, no satratoxin H added, and incubation in medium with serum for 72 h; *lane 2*, *3*, and *4*, satratoxin H in medium with serum for 24, 48, and 72 h, respectively; *lane 5*, no satratoxin H added, and incubation in serum-free medium for 72 h; *lane 6*, *7*, and *8*, satratoxin H in serum-free medium for 24, 48, and 72 h, respectively. The DNA was extracted after PC12 cells were treated with satratoxin H in serum-free medium or in medium with serum for 24, 48, and 72 h. The result shown was representative of three separated experiments.

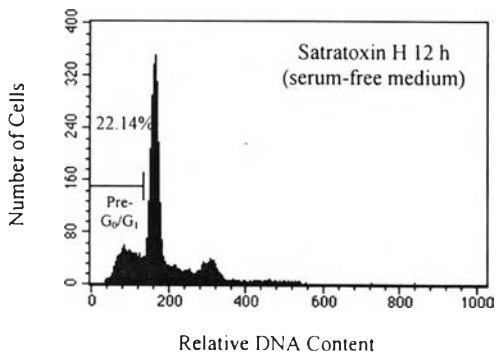
2.3 Flow cytometric analysis for measurement of subG$_1$ phase

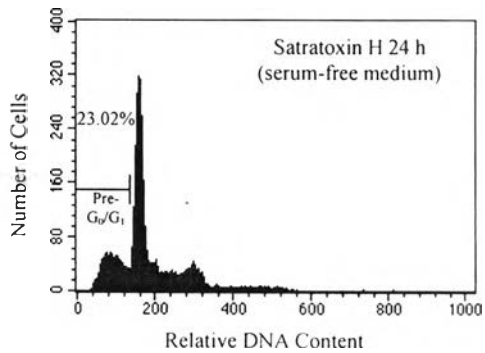
To confirm whether satratoxin H-induced cell death via apoptosis, the percentage of apoptotic cells was measured by flow cytometric analysis, after staining with PI which stains DNA by intercalating between the bases with a stoichiometry of one dye per 4-5 base pairs of DNA. The viable cells have normal amounts of DNA while cells that are undergoing late apoptosis have less DNA. The subG$_1$ peak in flow cytometry detection is considered as an indicator of cell apoptosis as shown in a representative result (Figure 9A). The results showed that the percentage of apoptotic cells (subG$_1$ peak) increased significantly (P<0.05) from 13.89 ± 0.31% in control to 20.48 ± 1.42% in the cells exposed to 50 nM satratoxin H in serum-free medium for 24 h (Figure 9B). However, when cells were exposed to satratoxin H for 36 h and 48 h, the population of apoptotic cells decreased to 18.52 ± 1.06 and 17.44 ± 2.2%, respectively. In contrast, the percentage of apoptotic cells in medium with serum increased significantly (P<0.01) from 0.83 ± 0.19% in control to 7.08 ± 2.68% in the cells exposed to 50 nM satratoxin H for 48 h (Figure 9B). Satratoxin H significantly increased (P<0.05) apoptotic cell death after 48-h incubation. The result showed that satratoxin H-treated PC12 cells incubated in medium with serum significantly increased DNA fragmentation after incubation for 48 h, meanwhile 24-h incubation in serum-free medium significantly increased the percentage of apoptosis then decreased after 48-h incubation. This result was similar to the data by agarose gel electrophoresis that the apoptotic band clearly saw after 24-h incubation of satratoxin H in serum-free medium then the apoptotic band gradually decreased at 48-h incubation and difficult to observe the band after 72-h incubation, meanwhile incubation of satratoxin H in medium with serum took more time to induce apoptosis. The results inferred that serum-free medium, resulted in a beneficial in the quiescence stage (G_0) of the cell cycle (Hulleman and Boonstra, 2001), may attribute PC12 cells more sensitive to satratoxin H than the cells incubated in medium with serum. From the results of apoptosis, PC12 cells were therefore incubated in serum-free medium during treatment of satratoxin H throughout the present study as shown in the previous reports (Panet *et al.*, 2001; Yoshizumi *et al.*, 2002).

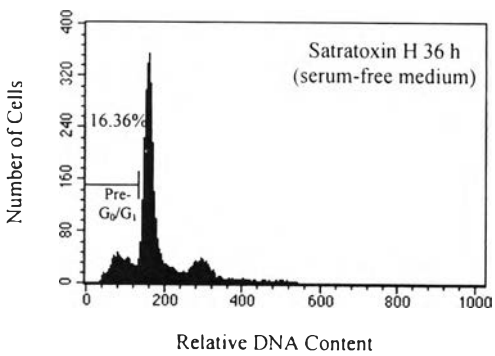
(A)

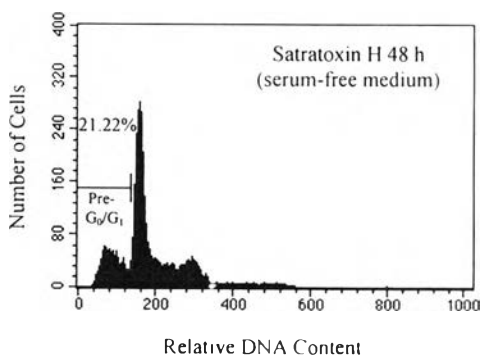

Control
(medium with serum)
G_0/G_1
1.86%
Pre-
G_0/G_1
S
G_2/M
Number of Cells
Relative DNA Content


Control
(serum-free medium)
15.8%
Pre-
G_0/G_1
Number of Cells
Relative DNA Content


Satratoxin H 12 h
(serum-free medium)
22.14%
Pre-
G_0/G_1
Number of Cells
Relative DNA Content


Satratoxin H 24 h
(serum-free medium)
23.02%
Pre-
G_0/G_1
Number of Cells
Relative DNA Content


Satratoxin H 36 h
(serum-free medium)
16.36%
Pre-
G_0/G_1
Number of Cells
Relative DNA Content


Satratoxin H 48 h
(serum-free medium)
21.22%
Pre-
G_0/G_1
Number of Cells
Relative DNA Content

(B)

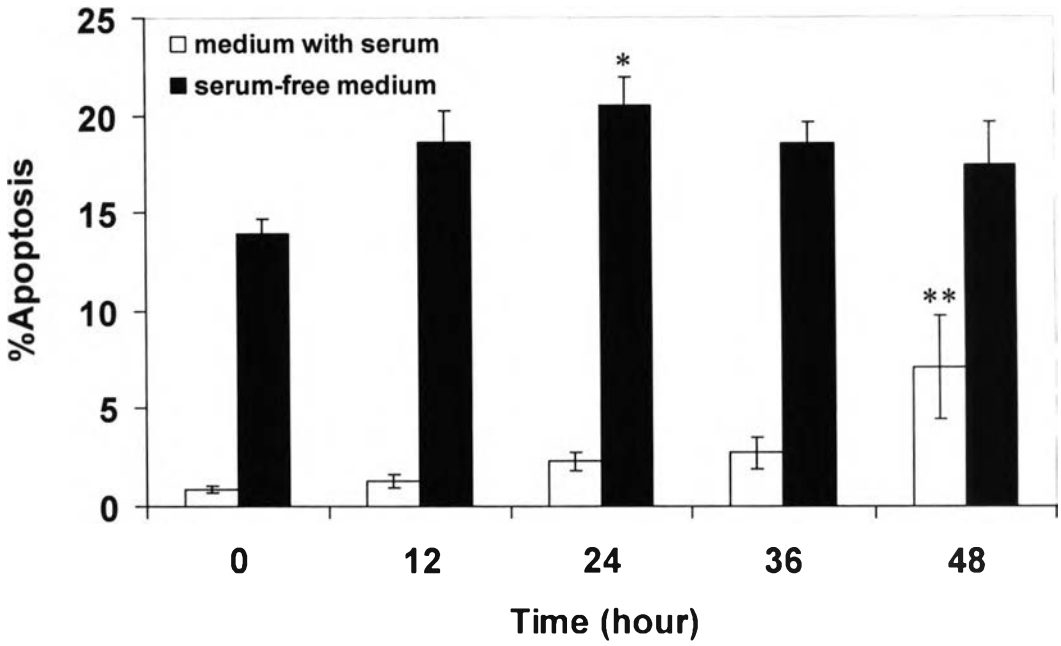


Figure 9 Flow cytometric analysis showing the effects of satratoxin H-induced apoptosis in PC12 cells. PC12 cells were treated with satratoxin H at a concentration of 50 nM for 12, 24, 36 and 48 h. The cells were exposed to medium with serum or serum-free medium throughout the experiment. Apoptotic cell death was assessed according to percent of cells with pre-G_0/G_1 peak as representative histogram of satratoxin H-treated PC12 cells in serum-free medium for 12, 24, 36 and 48 h (A). Bars indicated time points of satratoxin H-induced apoptosis incubated both in medium with serum and serum-free medium (B). Each point represents the means±SEM of four and five independent experiments in medium with serum and serum-free medium, respectively. $*P<0.05$, $**P<0.01$, compared with corresponding control group.

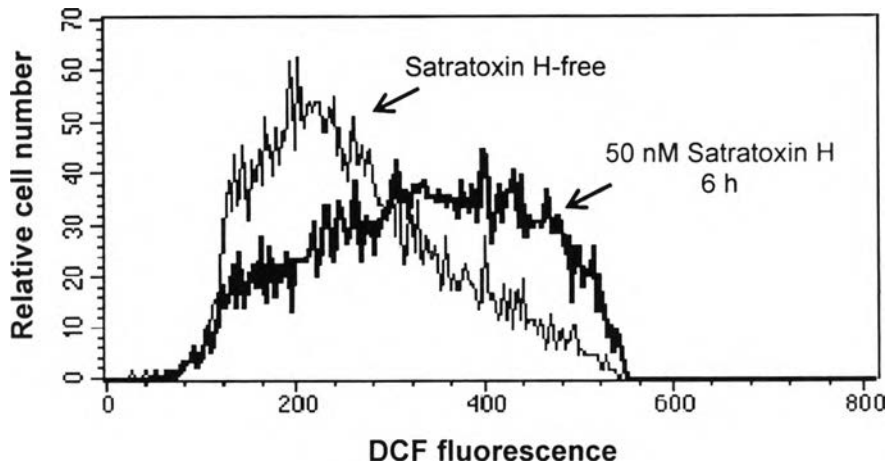
3. Satratoxin H-induced apoptosis via the generation of ROS and lipid peroxidation

Recently, it has been demonstrated that trichothecene mycotoxin AETD-induced apoptosis was associated with the production of intracellular ROS in human promyelocytic HL-60 cells (Pae *et al.*, 2003). In addition, trichothecene has been shown to generate lipid peroxidation in rat brain (Rizzo *et al.*, 1994). To elucidate whether oxidative stress mediated satratoxin H-induced apoptosis, ROS and lipid peroxidation were evaluated. The intracellular ROS level was determined by using DCFH-DA and detected by flow cytometry, and lipid peroxidation was evaluated by TBARS which gave the level of the total amount of lipid hydroperoxides and lipid aldehydes including MDA and 4-HNE derived from lipid peroxidation.

3.1 Satratoxin H-induced apoptosis via the generation of ROS

In order to further investigate the participation of oxidative stress in satratoxin H-induced apoptosis, ROS production was measured by flow cytometer using DCFH-DA. Exposing PC12 cells to satratoxin H at a concentration of 50 nM in serum-free medium increased the intracellular ROS level in time-dependent manner. Satratoxin H resulted in small increase the fluorescence at 3 h and significant increase in the fluorescent intensity at 6 h ($P<0.05$) then sustained the level until 24 h, indicating that satratoxin H increased ROS production (Figure 10). Since, the generation of ROS was coincident with apoptotic cell death. The result suggested that satratoxin H induced apoptosis in PC12 cells through the generation of ROS. An increased formation of ROS has been demonstrated to promote the apoptotic cell death, may be partially by depleting cellular antioxidants especially GSH (Pae *et al.*, 2003), and by inducing direct damage to DNA, proteins and lipids (Higuchi, 2004).

(A)



(B)

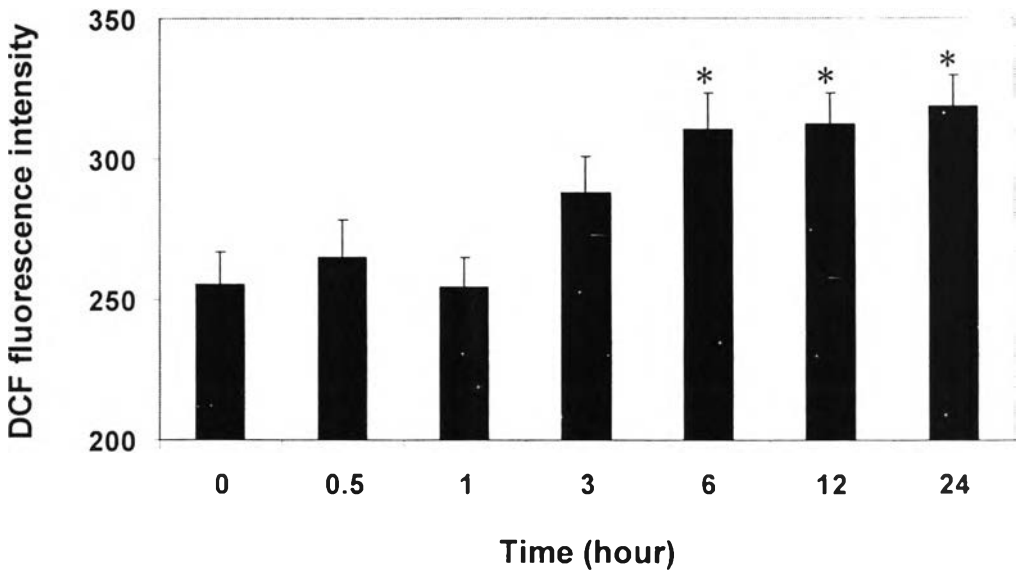


Figure 10 Time-dependent ROS formation in satratoxin H-treated PC12 cells. (A) Relative fluorescence intensity in PC12 cells was quantitated by a flow cytometry using DCFH-DA. Cells were incubated with satratoxin H-free medium (light peak) or 50 nM satratoxin H for 6 h (solid peak). (B) Cells were incubated 50 nM satratoxin H for indicated times. DCFH-DA was added to the culture medium 30 min before analysed by means of flow cytometry. Quantification of the relative fluorescent intensity from six independent experiments was performed, and results were expressed as the mean±SEM. $*P < 0.05$ compared to the control cells.

3.2 Satratoxin H increased lipid peroxidation in PC12 cells

Trichothecenes have been shown to induce lipid peroxidation in various tissues such as liver, spleen, and brain (Atroshi *et al.*, 1995; Rizzo *et al.*, 1994). Recently, it has been reported that ROS was closed relationship to the production of lipid peroxidation (Yang *et al.*, 2003). To elucidate whether the production of lipid peroxidation mediated satratoxin H-induced apoptosis in PC12 cells, the amounts of lipid peroxidation were evaluated by TBARS. Satratoxin H-treated cells in serum-free medium increased the TBARS production in time dependency and significantly increased the MDA concentration ($P<0.01$) after 36-h incubation as compared to control (serum-free) as shown in Figure 11. Taken together with the result of early and late apoptosis detected by Hoechst 33342 which apoptosis occurred after 24-h incubation, and the results of late apoptosis detected by agarose gel electrophoresis and flow cytometer using PI which apoptosis occur after 24-h incubation then gradually decreased as shown in Figure 7-9. Lipid peroxidation, a marker of necrosis (Higuchi, 2004), occurred after 36-h incubation which DNA ladder decreased (lanes 7, 8 in Figure 8), the results suggested that the capacity to generate lipid peroxidation in PC12 cells by satratoxin H may exert necrotic cell death, but not apoptosis.

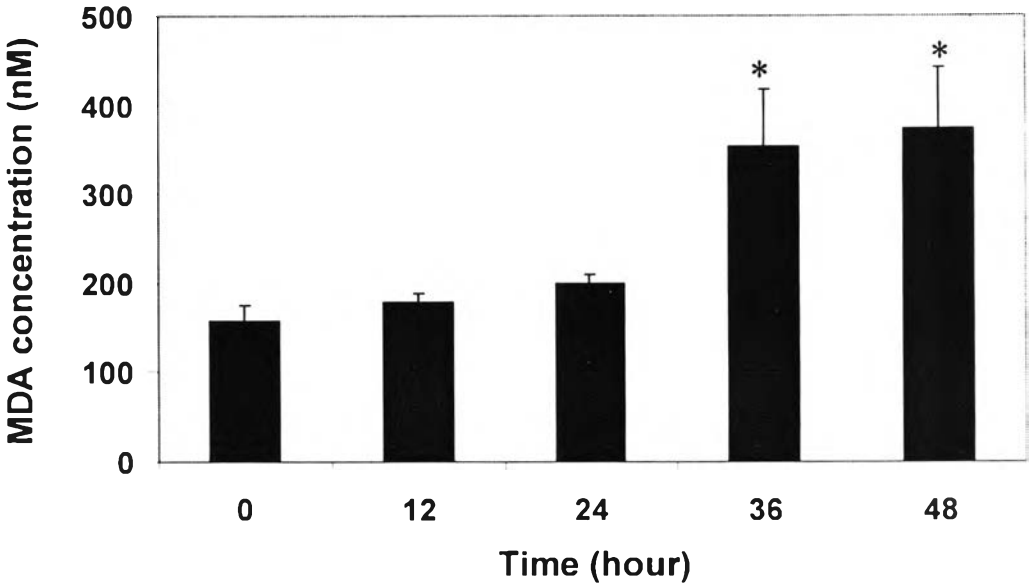


Figure 11 Effect of satratoxin H treatment on the production of MDA in PC12 cells. Time-dependency, 12, 24, 36, and 48 h, of satratoxin H (50 nM)–induced the production of lipid peroxides was done. Data were expressed as mean values, mean±SEM, n=4. *P<0.01 vs. control without satratoxin H incubated in serum-free medium.

4. Mechanistic study of satratoxin H-induced apoptosis via the activation of MAPKs

4.1 Satratoxin H-induced phosphorylation of p38 MAPK, JNK and ERK1/2

The preceding results showed that satratoxin H induced apoptosis was accompanied by the generation of ROS and the production of lipid peroxidation. p38 MAPK and JNK as well as ERK1/2 have been demonstrated to play a central role in ROS-induced apoptosis in numerous system models including PC12 cells (Kang *et al.*, 1998; Seo, *et al.*, 2001; Shin *et al.*, 2004). In addition, trichothecene mycotoxins have been reported to increase the ROS generation as well as activation of p38 MAPK, JNK, and ERK1/2 (Pae *et al.*, 2003; Yang *et al.*, 2000). Western blot analysis was performed to investigate the activation of p38 MAPK, JNK, as well as ERK1/2. To clarify the molecular mechanism of satratoxin H-induced apoptosis, the activation of MAPKs, time- and concentration-dependency, was examined in PC12 cells exposed to satratoxin H performed by Western blot analysis. The activation of p38 MAPK, JNK as well as ERK1/2 in satratoxin H-treated PC12 cells was observed. The activation of phosphorylated p38 MAPK, JNK and ERK1/2 increased in a time-dependent manner from 0.5 to 6 h after exposure to 50 nM satratoxin H (Figure 12A). Satratoxin H activated ERK1/2 via phosphorylation occurred early in 30 min and was sustained up to 6 h. Likewise, PC12 cells exposed to 50 nM satratoxin H resulted in the increase of the p38 MAPK activity at 3 h then maintained that level until 6 h. The weak phosphorylation of JNK was also induced by satratoxin H. Satratoxin H treatment had no effect on the levels of total unphosphorylated forms of p38 MAPK, JNK, and ERK1/2 presented in PC12 cells. The treatment with various concentrations (5-100 nM) of satratoxin H for 3 h stimulated the phosphorylation of p38 MAPK, JNK and ERK1/2 in a concentration-dependent manner (Figure 12B). As shown in Figure 10, the production of ROS gradually increased after 3-h incubation and significantly increased after 6-h incubation of satratoxin H. Taken together with the present data implied that ROS as well as p38 MAPK, JNK, and ERK1/2 appeared to be involved in the responses of satratoxin H-treated PC12 cells.

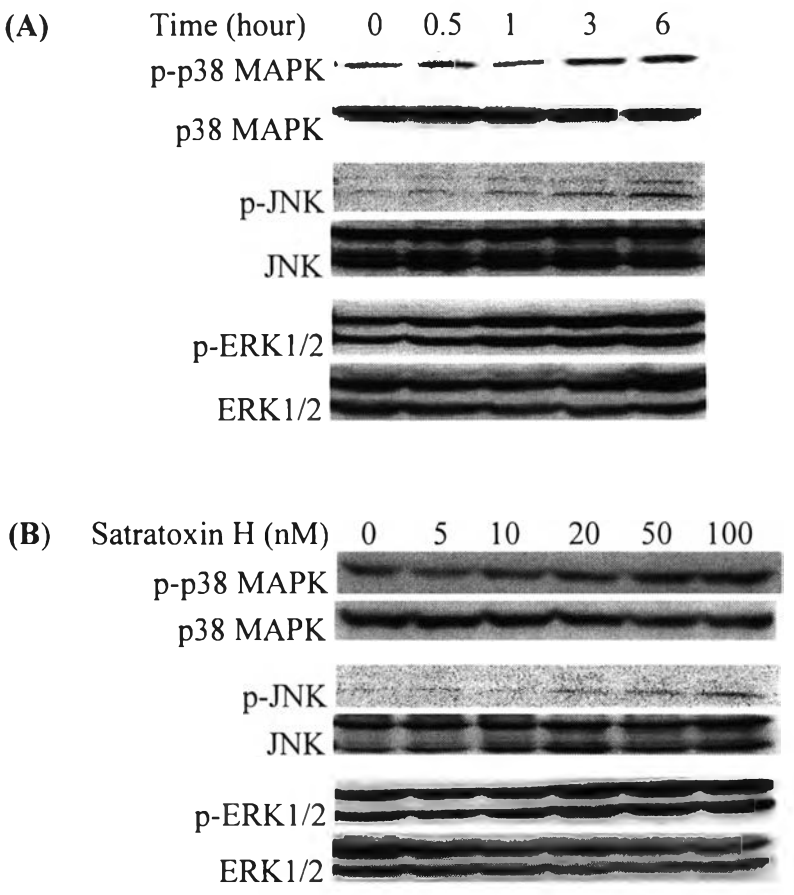


Figure 12 Satratoxin H-induced phosphorylation of p38 MAPK, JNK, and ERK1/2 in PC12 cells. (A) Time course of phosphorylation of these MAPKs after an exposure to 50 nM satratoxin H in serum-free medium for indicated time. (B) Concentration-dependency of satratoxin H-induced phosphorylation of MAPKs in the cells incubated with satratoxin H in serum-free medium for 3 h. Cell lysates (50 μg protein/lane) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Immunoblotting was performed using antibodies for non-phosphorylated or phosphorylated (p-) MAPKs. Representative result was shown, and data represented similar results in three separated experiments.

4.2. Effects of MAPK inhibitors on cell viabibity of satratoxin H-treated cells

To verify the role of p38 MAPK, JNK, and ERK1/2 pathway in satratoxin H-induced apoptosis in PC12 cells, cells were pretreated with 30 μM p38 inhibitor SB203580, or 10 μM JNK inhibitor SP600125 or 30 μM ERK inhibitor PD98059 for 1 h before exposure to satratoxin H (1-100 nM) for 48 h. Cell viability was measured by MTT reduction assay. p38 inhibitor SB203580 at a concentration of 30 μM and JNK inhibitor SP600125 at a concentration of 10 μM significantly increase cell viability from 33.01 ± 9.35 to 57.61 ± 4.65, and to 53.32 ± 16.32, respectively, meanwhile PD98059 at a concentration of 30 μM did not affect the cell viability (Figure 13). Incubation with SB203580 at a concentration of 30 μM, PD98059 at a concentration of 30 μM or SP600125 at a concentration of 10 μM alone showed little effect on cell viability. These results indicated that the role of p38 MAPK and JNK, in part, contributed to satratoxin H-induced apoptosis in PC12 cells.

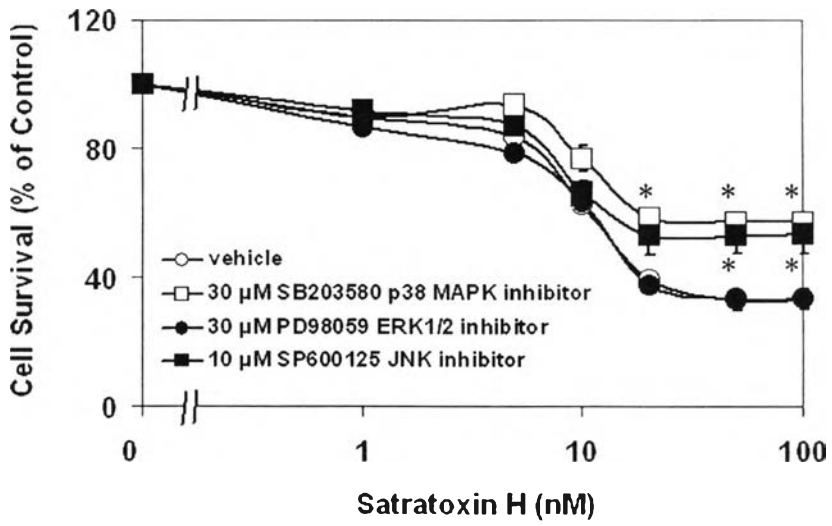


Figure 13 Effects of SB203580 at 30 μM, PD98059 30 μM and SP600125 10 μM on satratoxin H-induced cytotoxicity. Inhibitors were added 1 h before exposure to various concentrations of satratoxin H, then the cells were further incubated for 48 h. Data were normalized to control values, which were set to 100%. Each point represented the means±SEM of three independent experiments, each performed in triplicate. *P<0.05 significantly different from the cells exposed to satratoxin-H alone (vehicle group).

4.3 Effects of MAPK inhibitors on satratoxin H-induced p38 MAPK, JNK, and ERK1/2 activation

To verify the effectiveness of p38 kinase inhibitor SB203580, JNK inhibitor SP600125, and ERK1/2 inhibitor PD98059 on the activation of satratoxin H-induced p38 MAPK, JNK, and ERK1/2 phosphorylation, respectively, PC12 cells treated with inhibitors and/or satratoxin H were prepared for Western blot analysis. Pretreatment of cells with SB203580 (30 μM), SP600125 (10 μM), or PD98059 (30 μM) for 1 h before exposure to 50 nM satratoxin H for another 6 h, which showed the highest activation of p38 MAPK, JNK, and ERK1/2 in time-course study as shown in Figure 12A, was used in this experiment. The result was correlated well with the result of inhibitors detected by MTT assay as shown in Figure 13, that incubation of SB203580 or PD98059 alone decreased the phosphorylated p38 MAPK and ERK1/2, respectively (Figure 14). In addition, pretreatment of SB203580 followed by the addition of satratoxin H for 6 h reduced the phosphorylation of p38 MAPK, and pretreatment of PD98059 ERK1/2 inhibited the phosphorylation of ERK1/2. Since the weak phosphorylation of JNK in satratoxin H-treated PC12 cells, the effect of SP600125 treatment was not clearly seen (data not shown). Therefore, SB203580 and PD98059 were verified to be effective by inhibiting satratoxin H-induced p38 MAPK and ERK1/2 activation, respectively. The results supported that satratoxin H induced apoptosis through the activation of p38 MAPK pathway.

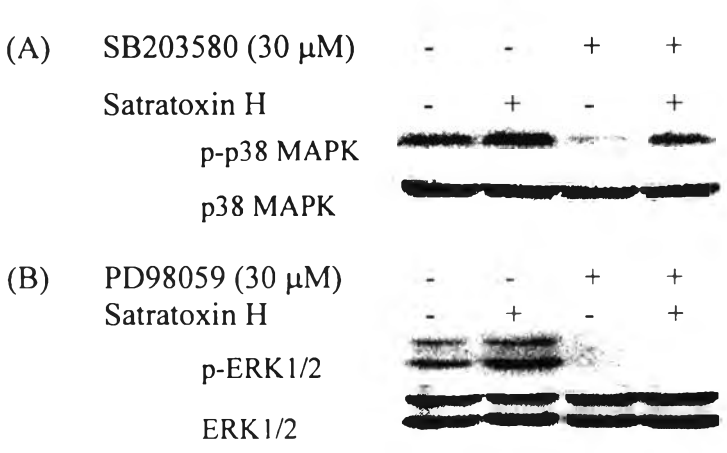


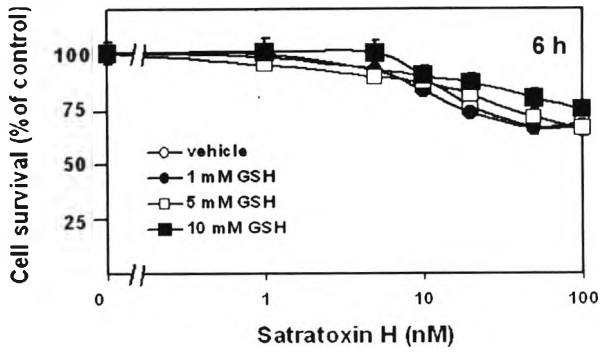
Figure 14 Effect of p38 inhibitor SB203580 (A), and ERK1/2 inhibitor PD98059 (B) on satratoxin H-induced p38 MAPK, and ERK1/2 activation. Cells were pretreated with SB203580 (30 μM) or PD98059 (30 μM) for 1 h before the addition of satratoxin H (50 nM). Cells were collected after 6-h incubation of satratoxin H and analyzed MAPKs by Western blot analysis.

5. Protective effect of antioxidant(s) on satratoxin H-treated PC12 cells

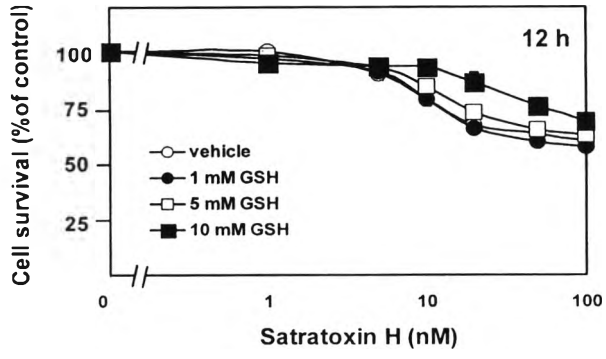
5.1 Effect of antioxidants on satratoxin H-induced cytotoxicity.

Toxins including AETD-induced the generation of ROS have been shown to protect apoptosis by antioxidants NAC and GSH (Li *et al.*, 2003; Lizard *et al.*, 1998; Pae *et al.*, 2003; Shimizu *et al.*, 2002) as well as trolox (Ameen *et al.*, 2004). It has been reported that GSH was found in various mammalian tissues in a physiological range of 0.5-10 mM (Dringen, 2000). Meanwhile, vitamin E at a dose of less than 1 mM was not toxic to cells (Cao and Cutler, 1993). Therefore, GSH (1, 5, and 10 mM) and trolox (0.01, 0.1 and 1 mM) were used in the present study. NAC, a precursor of intracellular GSH synthesis (Yan *et al.*, 1995), penetrates across cell membrane and has been used in numerous experiments ranging between 1-15 mM (Capella *et al.*, 2002; Li *et al.*, 2003; Lizard *et al.*, 1998; Shimizu *et al.*, 2002). To determine whether the toxic effect of satratoxin H was protected by extracellular GSH or intracellular GSH, NAC (1, 5, and 10 mM) was used in the present study. The inhibitory effect of ROS scavengers: GSH, NAC, and trolox on satratoxin H-induced apoptosis were initially determined by MTT assay. Antioxidants for treatment were freshly prepared in medium from stock solution. The results showed that incubation of GSH (1, 5, and 10 mM) or NAC (1, 5 mM, and 10 mM) for 6-24 h, or incubation of trolox (0.01, 0.1 and 1 mM) for 24-48 h, did not exert a protective effect against satratoxin H-induced apoptosis as shown in Figures 15A-D, 15E-H, 15I-K, respectively. Antioxidant alone did not affect cell viability as shown in a representative result at 24 h (Figure 15L). The result suggested that satratoxin H-induced the generation of ROS might not be the major cause of cell death in PC12 cells which has been reported in myeloid cells induced by trichothecene mycotoxins (Shifrin and Anderson, 1999; Uzarski *et al.*, 2003; Yang *et al.*, 2000).

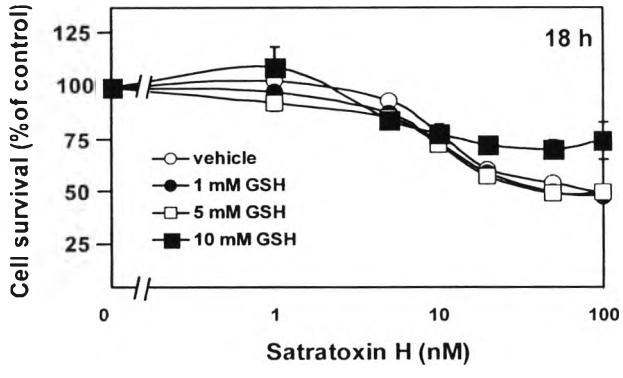
(A)


6 h
Cell survival (% of control)
100
75
50
25
0
1
10
100
Satratoxin H (nM)
vehicle
1 mM GSH
5 mM GSH
10 mM GSH

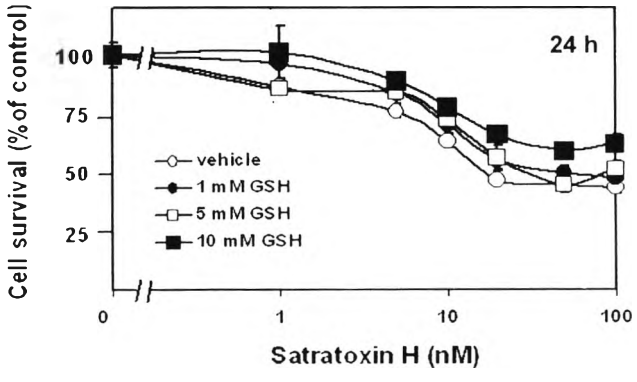
(B)


12 h
Cell survival (% of control)
100
75
50
25
0
1
10
100
Satratoxin H (nM)
vehicle
1 mM GSH
5 mM GSH
10 mM GSH

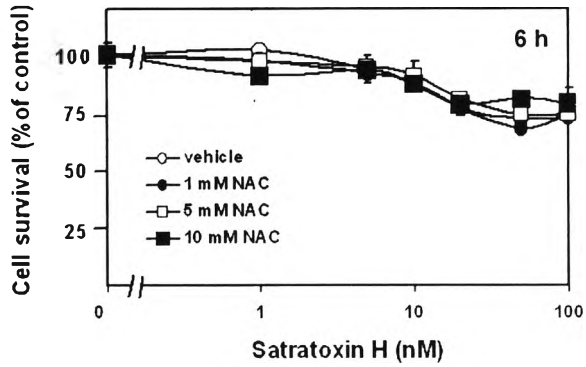
(C)


18 h
Cell survival (% of control)
125
100
75
50
25
0
1
10
100
Satratoxin H (nM)
vehicle
1 mM GSH
5 mM GSH
10 mM GSH

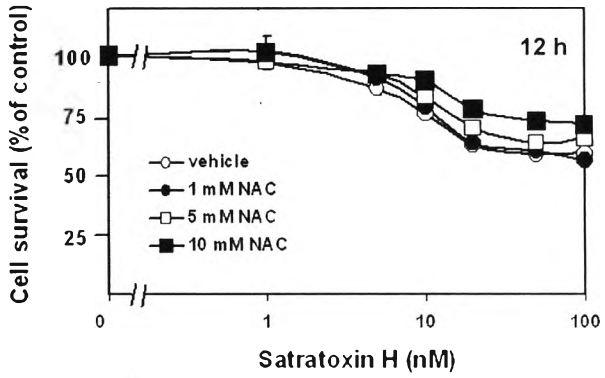
(D)


24 h
Cell survival (% of control)
100
75
50
25
0
1
10
100
Satratoxin H (nM)
vehicle
1 mM GSH
5 mM GSH
10 mM GSH

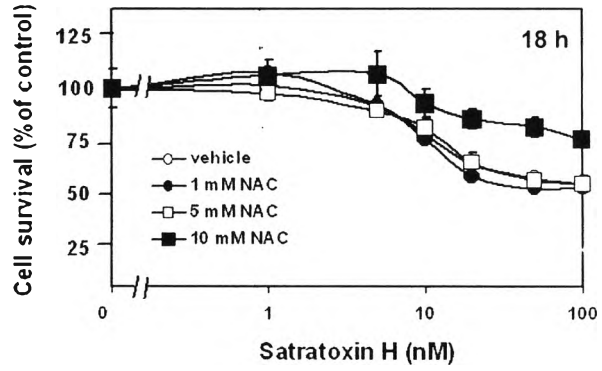
(E)


6 h
Cell survival (% of control)
100
75
50
25
0
1
10
100
Satratoxin H (nM)
vehicle
1 mM NAC
5 mM NAC
10 mM NAC

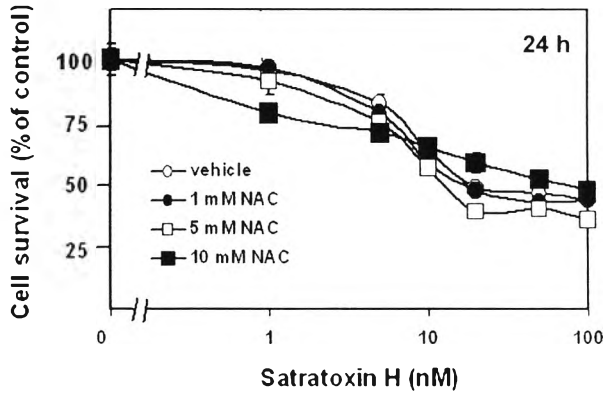
(F)


12 h
Cell survival (% of control)
100
75
50
25
0
1
10
100
Satratoxin H (nM)
vehicle
1 mM NAC
5 mM NAC
10 mM NAC

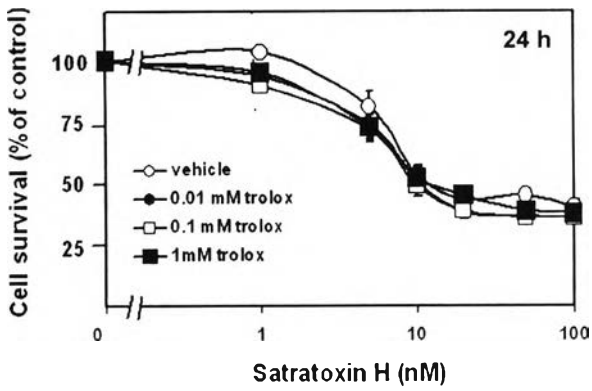
(G)


18 h
Cell survival (% of control)
125
100
75
50
25
0
1
10
100
Satratoxin H (nM)
vehicle
1 mM NAC
5 mM NAC
10 mM NAC

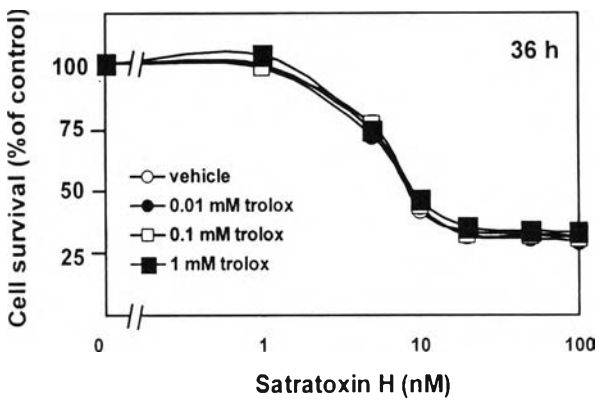
(H)


24 h
Cell survival (% of control)
100
75
50
25
0
1
10
100
Satratoxin H (nM)
vehicle
1 mM NAC
5 mM NAC
10 mM NAC

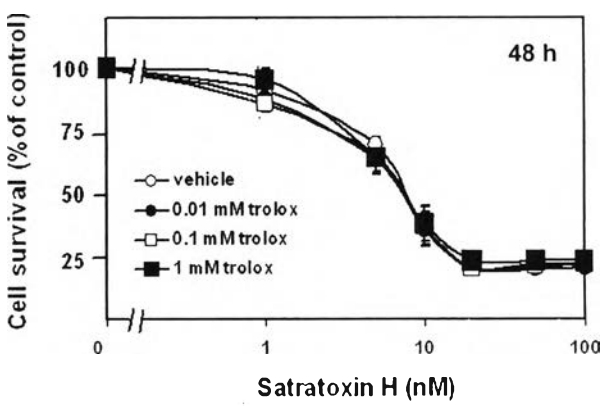
(I)


24 h
Cell survival (% of control)
100
75
50
25
0
1
10
100
vehicle
0.01 mM trolox
0.1 mM trolox
1mM trolox
Satratoxin H (nM)

(J)


36 h
Cell survival (% of control)
100
75
50
25
0
1
10
100
vehicle
0.01 mM trolox
0.1 mM trolox
1 mM trolox
Satratoxin H (nM)

(K)


48 h
Cell survival (% of control)
100
75
50
25
0
1
10
100
vehicle
0.01 mM trolox
0.1 mM trolox
1 mM trolox
Satratoxin H (nM)

(L)

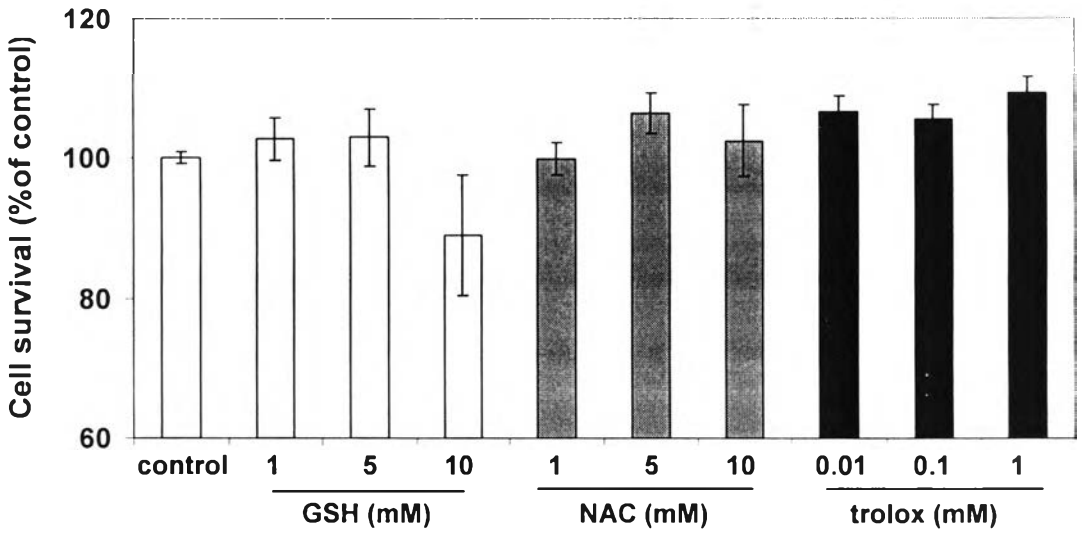


Figure 15 Effect of reduced glutathione (GSH), *N*-acetyl-L-cysteine (NAC) or 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) on satratoxin H-induced cytotoxicity in PC12 cells. Cells were treated with indicated concentrations of GSH at indicated time points (A-D), or NAC at indicated time points (E-H) and satratoxin H in serum-free medium for 6-24 h as well as trolox at indicated time points (I-K) and satratoxin H in serum-free medium for 24-48 h. All cells were exposure to serum-free medium for the same period of time. Antioxidant alone did not affect cell viability as shown in a representative result at 24-h incubation (L). Cell viability was determined by MTT assay. Data were normalized to control values, which were set to 100%. Each point represented mean±SEM of independent three experiments, each performed in triplicate.

5.2 Effect of antioxidants on satratoxin H-induced apoptosis

To confirm that satratoxin H-induced apoptosis could not be protected by antioxidants GSH and NAC as shown in Figure 15, and to determine whether the generation of ROS is essential for satratoxin H-induced apoptosis, satratoxin H-treated PC12 cells were coincubated with GSH or NAC in serum-free medium for 24 h and determined by flow cytometry using PI. Trolox has been well known to protect the propagation of lipid peroxidation (Cao and Cutler, 1993; Horáková *et al.*, 2003) which may not involve in satratoxin H-induced apoptosis in the present study (Figure 7-9, 11), only GSH and NAC were used. Since detection of cell viability of GSH- or NAC-treated cells by MTT assay which based on the function of mitochodrial dehydrogenase, the result was supported flow cytometry. The results showed that cotreatment of GSH (1, 5, and 10 mM) or NAC (1, 5, and 10 mM) and satratoxin H at a concentration of 50 nM did not significantly decrease the percentage of apoptosis when compared to satratoxin H-treated PC12 cells as shown in Figure 16. However, incubation of satratoxin H alone significantly increased ($P<0.05$) the percentage of apoptosis when compared to control (serum-free medium). Incubation of GSH or NAC alone did not affect DNA content of PC12 cells. Taken together with the results from MTT assay (Figure 15) and satratoxin H-induced the generation of ROS (Figure 10) suggested that the generation of ROS may be not the main pathway on satratoxin H-induced apoptosis in PC12 cells. Other oxidants may play an important role in satratoxin H-induced apoptosis in PC12 cells as shown by Rizzo *et al.*, 1994 that deoxynivalenol and T-2 toxin decreased GSH content, and the enzymatic responses of catalase, superoxide dismutase, and cytochrome P-450. Otherwise, another important pathway-induced apoptosis stimulated by trichothecenes AETD and T-2 toxins included mitochondrial pathway (Nagase *et al.*, 2001; Pae *et al.*, 2003) and protein inhibition (Shifrin and Anderson, 1999) should be considered on satratoxin-induced apoptosis.

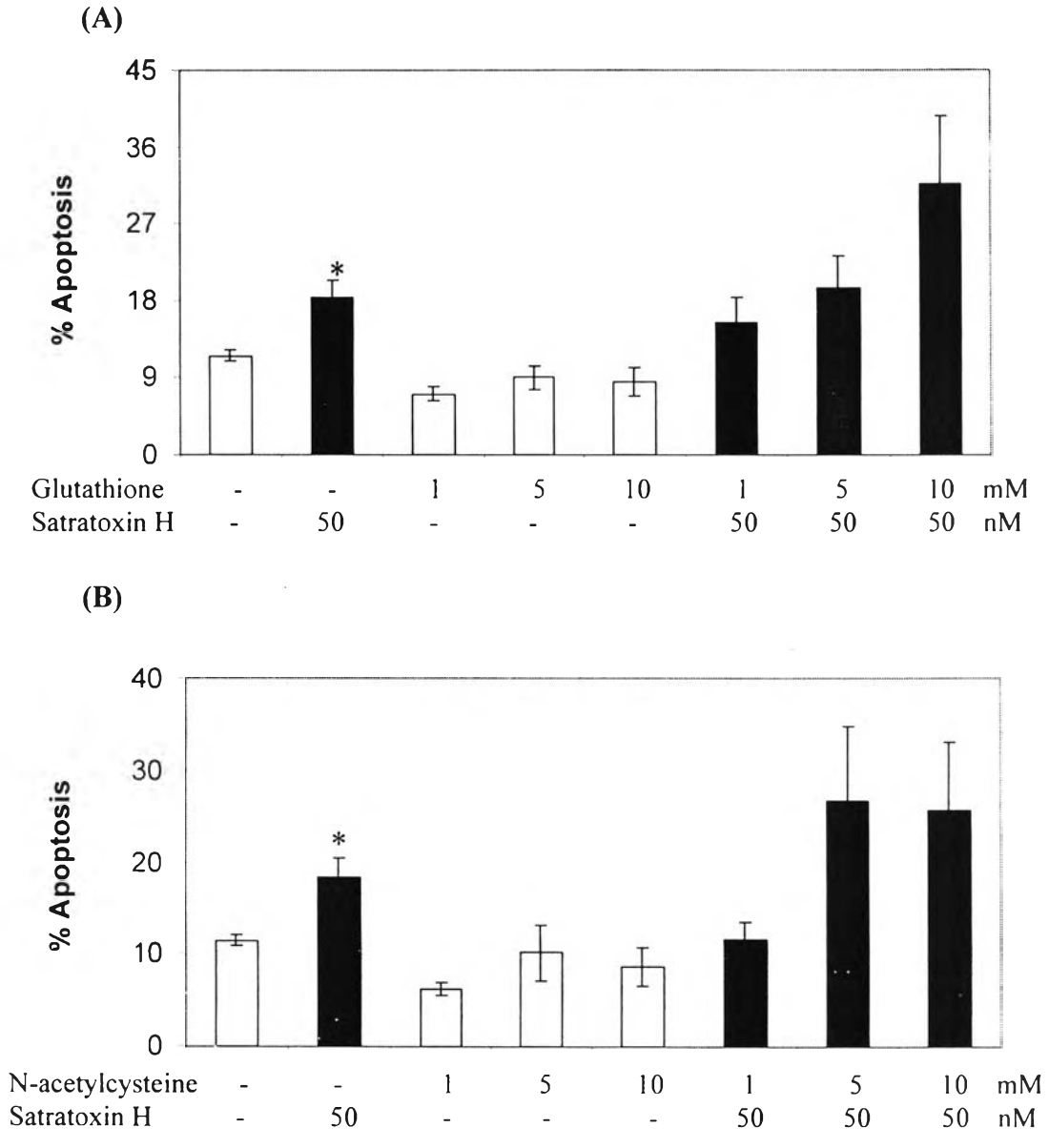


Figure 16 Flow cytometric analysis for the effects of antioxidants on satratoxin H-treated PC12 cells. Satratoxin H (50 nM) with or without antioxidants were incubated for 24 h. The percentage of apoptotic cells in the presence of antioxidants GSH (A) or NAC (B) alone as well as incubated with satratoxin H or satratoxin H alone was shown as bars. Data were expressed as mean values, mean±SEM, n=3. *P<0.05 vs. control without satratoxin H incubated in serum-free medium.

5.3 Effect of antioxidants on the generation of ROS in satratoxin H-treated PC12 cells

To determine whether the generation of ROS is essential for satratoxin H-induced apoptotic cell death as shown in the previous study (Lin *et al.*, 2003), satratoxin H-treated PC12 cells was further measured by using antioxidants. Since satratoxin H-induced the generation of ROS significantly increased at 6 h (Figure 10), cotreatment of GSH or NAC, and satratoxin H was therefore investigated by incubation for 6 h as well. Flow cytometry, together with DCFH-DA, was used to measure ROS generated by satratoxin H-stimulated PC12 cells. To overcome this potential problem, antioxidants GSH and NAC, were used with the dye staining. In addition, since free radicals have been shown to be the mediators of cell death, the effects of GSH and NAC on the protection of apoptosis induced by satratoxin H were evaluated. The results showed that PC12 cells treated with satratoxin H alone significantly increase intracellular ROS level when compared to the cells incubated in serum-free medium. Meanwhile, satratoxin H-induced the generation of ROS was decreased by the addition of antioxidants GSH (1 mM) (Figures 17), GSH (5 and 10 mM) and NAC (1, 5 and 10 mM) did not significantly decrease the production of ROS in satratoxin H-treated PC12 cells. The result suggested that GSH (1 mM) partially neutralized the generation of ROS from satratoxin H. GSH may decrease the number of satratoxin H extracellularly by binding to lactone ring (Whitton *et al.*, 2003). It has been shown that GSH transported across cell membrane through Na^+-independent GSH transporter in Cos-7 cells and through Na^+-dependent transporter in an immortalized mouse brain endothelial cells (Kannan *et al.*, 1999). Therefore, it was possible that GSH played an important role to decrease the generation of intracellular ROS. GSH, a specific scavenger of H_2O_2, significantly decreased the generation of ROS, the result provided the evidence of a possible role of ROS, especially H_2O_2, as a mediator induced apoptosis through the activation of p38 MAP kinase and JNK by satratoxin H in PC12 cells. Since NAC did not decrease the generation of ROS in the present study, it was possible that satratoxin H may affect the activity of γ-glutamylcysteine synthase and/or GSH synthase. NAC, a precusor of GSH synthesis, has been shown to increase intracellular GSH in concentration- and time-dependency in alveolar epithelial cells (Haddad, 2002).

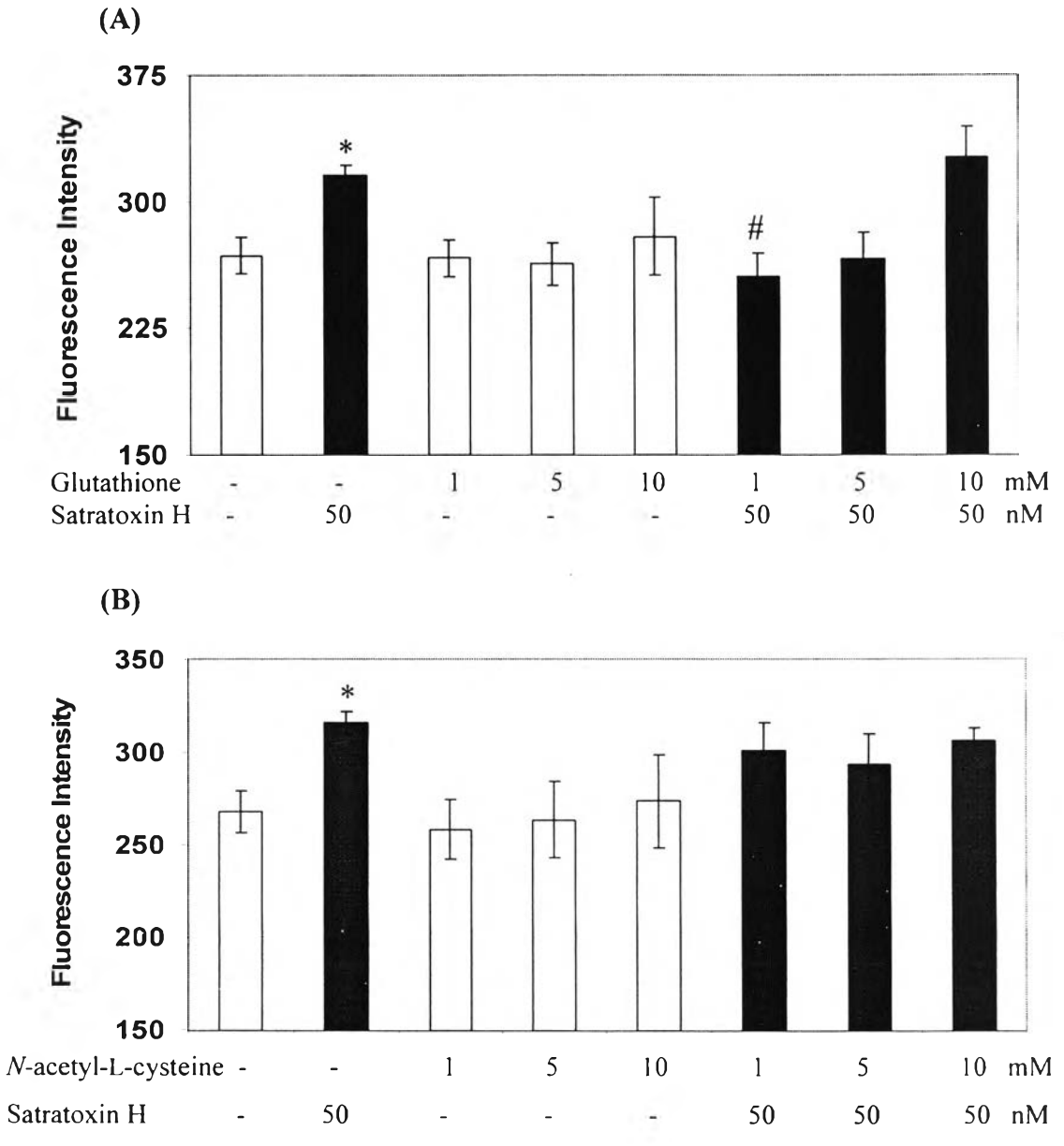


Figure 17 Effects of GSH (17A) and NAC (17B) on the generation of ROS induced by satratoxin H. PC12 cells was treated with satratoxin H (50 nM) and/or coincubated with GSH or NAC for 6 h. 2',7' dichlorofluorescein diacetate was added to the culture medium 30 min before analysed by mean of flow cytometry. Result showed typical histograms and statistic analysis of their means. Each point represented mean ± SEM of at least three independent experiments. $^{*}P<0.05$, significantly differed from the control group (no treatment). $^{\#}P<0.05$, significantly differed from 50 nM satratoxin H-treated group.

5.4 Effect of antioxidants on satratoxin H-induced the generation of lipid peroxidation in PC12 cells

It has been documented that vitamin E and GSH protected the production of lipid peroxidation by donated hydrogen atom to lipid peroxides and conjugated with lipid peroxides, respectively (Cao and Cutler, 1993; Yang *et al.*, 2003). Trolox is a water soluble vitamin E. To examine the ability of GSH and trolox to protect against the production of lipid peroxidation induced by satratoxin H, PC12 cells were treated simultaneously with reduced glutathione or trolox and satratoxin H. The production of lipid peroxides was significantly increased ($P<0.01$) in PC12 cells after exposure to satratoxin H (50 nM) for 36-48 h, when compared to control (serum-free medium). The data did not show statistically significant difference of the amount of lipid peroxidation in the PC12 cells exposed to 50 nM satratoxin H when cotreated with various concentrations of either GSH (1, 5, or 10 mM) or trolox (0.01, 0.1, or 1 mM) for 48 h. The amount of MDA did not change in the cells incubated with only GSH or trolox. The result revealed that GSH (1, 5, 10 mM) and trolox (0.01, 0.1, 1 mM) cannot protect cells from satratoxin H-induced the production of lipid peroxidation (Figure 18). The results revealed that antioxidants GSH and trolox did not protect the production of lipid peroxidation induced by satratoxin H-treated PC12 cells in the present condition.

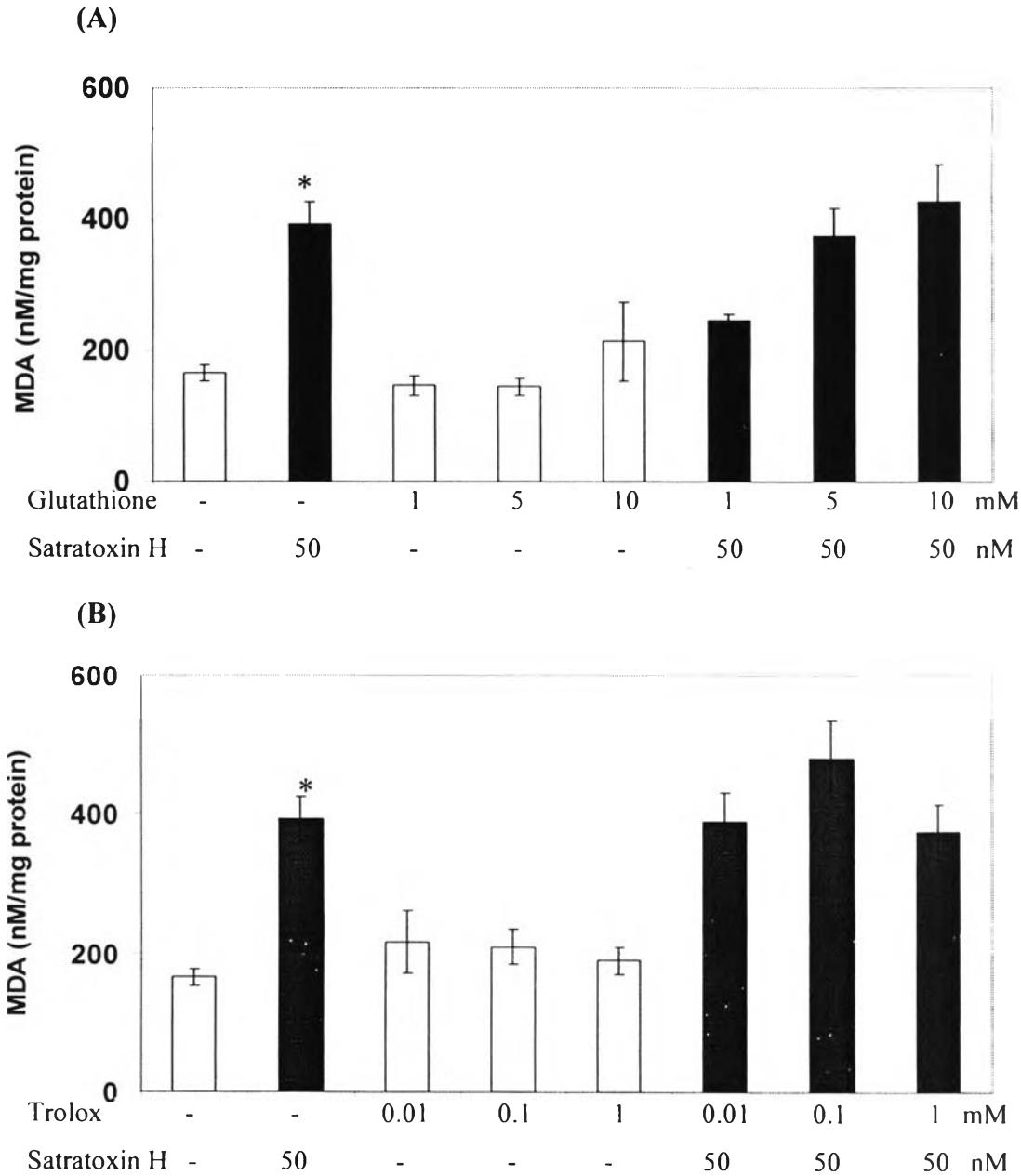


Figure 18 Influence of GSH (A), and trolox (B) on the production of lipid peroxides in satratoxin H-treated PC12 cells. Malondialdehyde (MDA) was measured photometrically after co-treatment of satratoxin H at a concentration of 50 nM and GSH (1, 5, and 10 mM) or trolox (0.01, 0.1, and 1 mM) in serum-free medium for 48 h. Data were expressed as mean values±SEM, control group and satratoxin-treated group, n=11; the rest, n=4. Significance between satratoxin H-treated PC12 cells and the control PC12 cells: $*P<0.01$, compared to control. No significant difference between GSH-treated group compared to the satratoxin H-treated control group.