CHAPTER V
RESULTS

5.1 Potential nitric oxide (NO) consumption in plasma

5.1.1 Potential nitric oxide (NO) consumption in plasma of chronical cadmium-exposed and normal subjects

To determine the ability of plasma of chronic cadmium-exposed and normal subjects, 50 µL of plasma was injected into purge vessel containing DETANONOate (50 µM) in PBS, 37^{o}C. The decrease of chemiluminescence signal of NO was monitored (Figure 5.1). The blood cadmium levels of control and cadmium-exposed subjects were 0.32 ± 0.13 and 8.2 ± 0.82 µg/L, respectively (mean ± SEM, n = 25 each, P = 0.0001 by Student's t test). The urinary cadmium levels of control and cadmium-exposed subjects were 1.13 ± 007 and 10.08 ± 1.01 µg/L, respectively (mean ± SEM, n = 25 each, P = 0.0001 by Student's t test). The levels of potential NO consumption by plasma of cadmium-exposed subjects were not different from control subjects (mean ± SEM, n = 25 each, 0.084 ± 0.01 µM versus 0.089 ± 0.013 µM, respectively; P > 0.5) (Figure 5.2). These results suggest that plasma NO consumption is an unlikely cause of decreased nitrite levels in cadmium-exposed subjects.

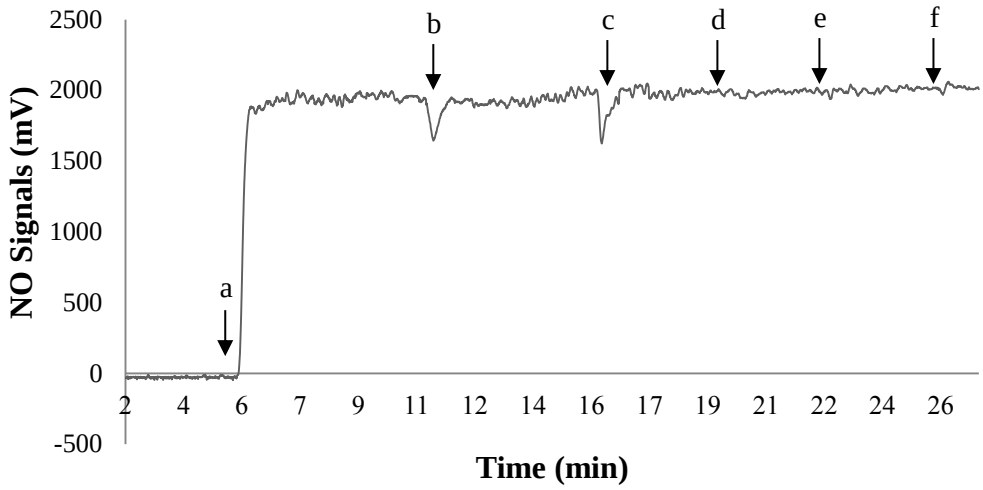

Figure 5.1 Trace chemiluminescence signal of NO released from 50 µM DETANONOate (a). Arrows indicate injection of plasma of control subjects (b), plasma of cadmium-exposed subjects (c), and $CdCl_2$ at concentrations of 0.1 (d), 1 (e), and 10 µM (f). Area under the curve of signal decay after injection was calculated and converted into NO concentration.

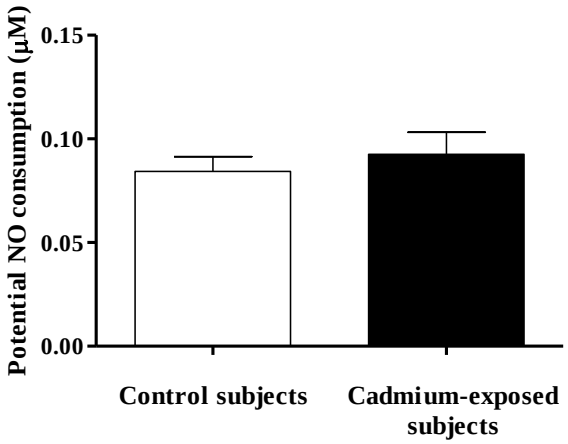

Figure 5.2 Potential NO consumption by plasma of control (subjects with urinary cadmium < 0.5 mg/g creatinine) and chronic cadmium-exposed subjects (subjects with urinary cadmium >5 mg/g creatinine). Values are means ± S.E.M. (n = 25 each, Student's t test)

5.2 Endothelial dysfunction

5.2.1 Assessment of the endothelial dysfunction in cadmium-exposed and control plasma subjects

Further, we measured soluble thrombomodulin (a marker of endothelial dysfunction) in plasma of cadmium-exposed and control subjects using thrombomodulin ELISA kits (Abcam, UK). The levels of thrombomodulin were higher in cadmium-exposed than control subjects: 13.42 ± 1.0 vs. 6.09 ± 0.3 ng/mL, respectively (Figure 5.3.). These results suggest that chronic cadmium exposure increased the release of TM into plasma representing endothelial dysfunction.

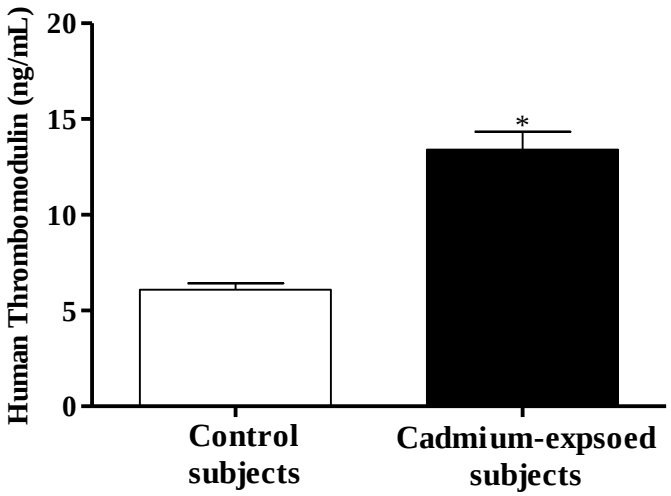

Figure 5.3 The levels of thrombomodulin (TM) in control and cadmium-exposed subjects (n = 15 each). Values are means ± SEM. *$P < 0.05$ (Student's t test).

5.3 Correlation

5.3.1 Correlation between plasma thrombomodulin and clinical parameters

The results of correlation analysis between plasma thrombomodulin and clinical parameters were analyzed by Pearson method. Plasma thrombomodulin in plasma correlated positively with urine cadmium (n = 39, r = 0.4127, P = 0.009) and whole blood cadmium (n = 39, r = 0.3181, P = 0.0484) (Figure 5.4 and 5.5). However, there was no significant correlation between plasma thrombomodulin and nitrite levels (n = 39, r = -0.1007, P = 0.5420) (Figure 5.6).

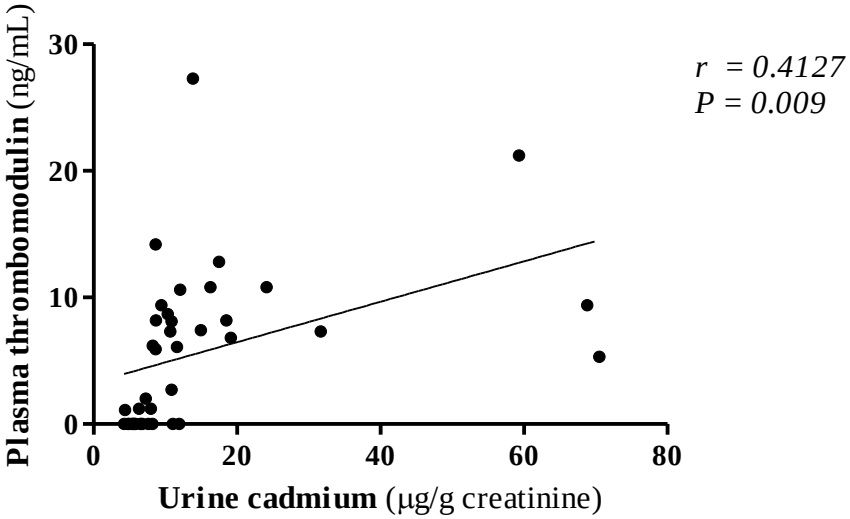

Figure 5.4 Correlation between soluble thrombomodulin and urine cadmium. Correlation was analyzed by Pearson's method.

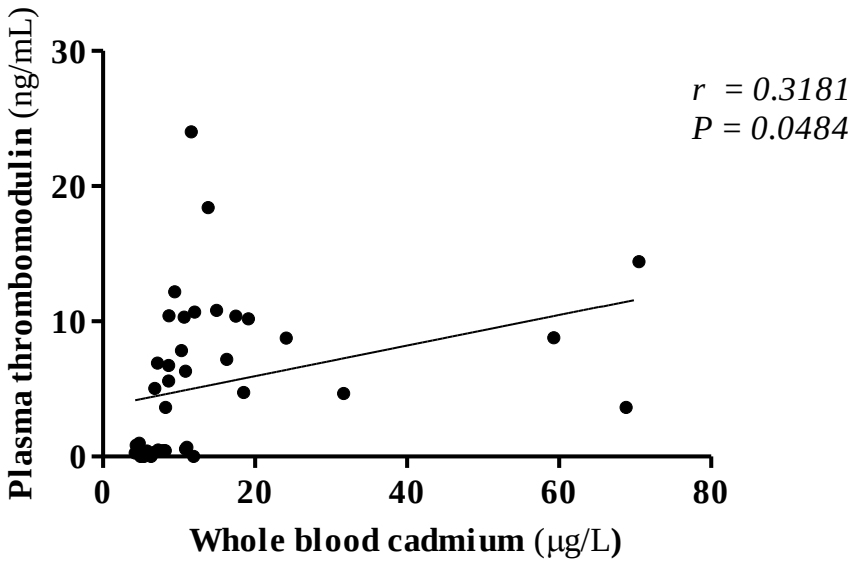

Figure 5.5 Correlation between plasma thrombomodulin and the levels of whole blood cadmium. Correlation was analyzed by Pearson's method.

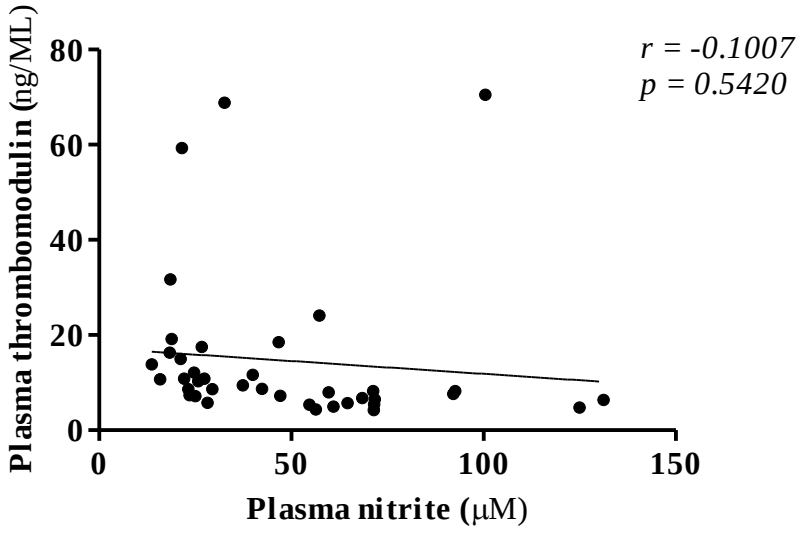

Figure 5.6 Correlation between plasma thrombomodulin and plasma nitrite. Correlation was analyzed by Pearson's method.

5.4 Cadmium cytotoxicity

5.4.1 Assessment of cadmium cytotoxicity on HCAEC

HCAECs cell viability in the presence of increasing concentrations of cadmium was assessed using MTT assays. The assessment of HCAECs cell viability was studied following incubation of cells with different concentrations of cadmium for 6, 24 and 48 h. After incubated for 6 and 24 h, cell viability was decreased to 18.8% and 7.9%, respectively at cadmium concentration of 300 µM (Figure 5.7A and B). After 48 h of incubation, cell viability was decreased to 5.6% and 2.4 % at Cd concentration of 100 and 300 µM, respectively (Figure 5.7C). The 50% lethal concentrations (LC_{50}) of cadmium on HCAECs were 134.8 ± 2.2, 116.2 ± 1.4 and 63.5 ± 1.1 µM following 6, 24 and 48 h of incubation (Figure 5.7D), respectively

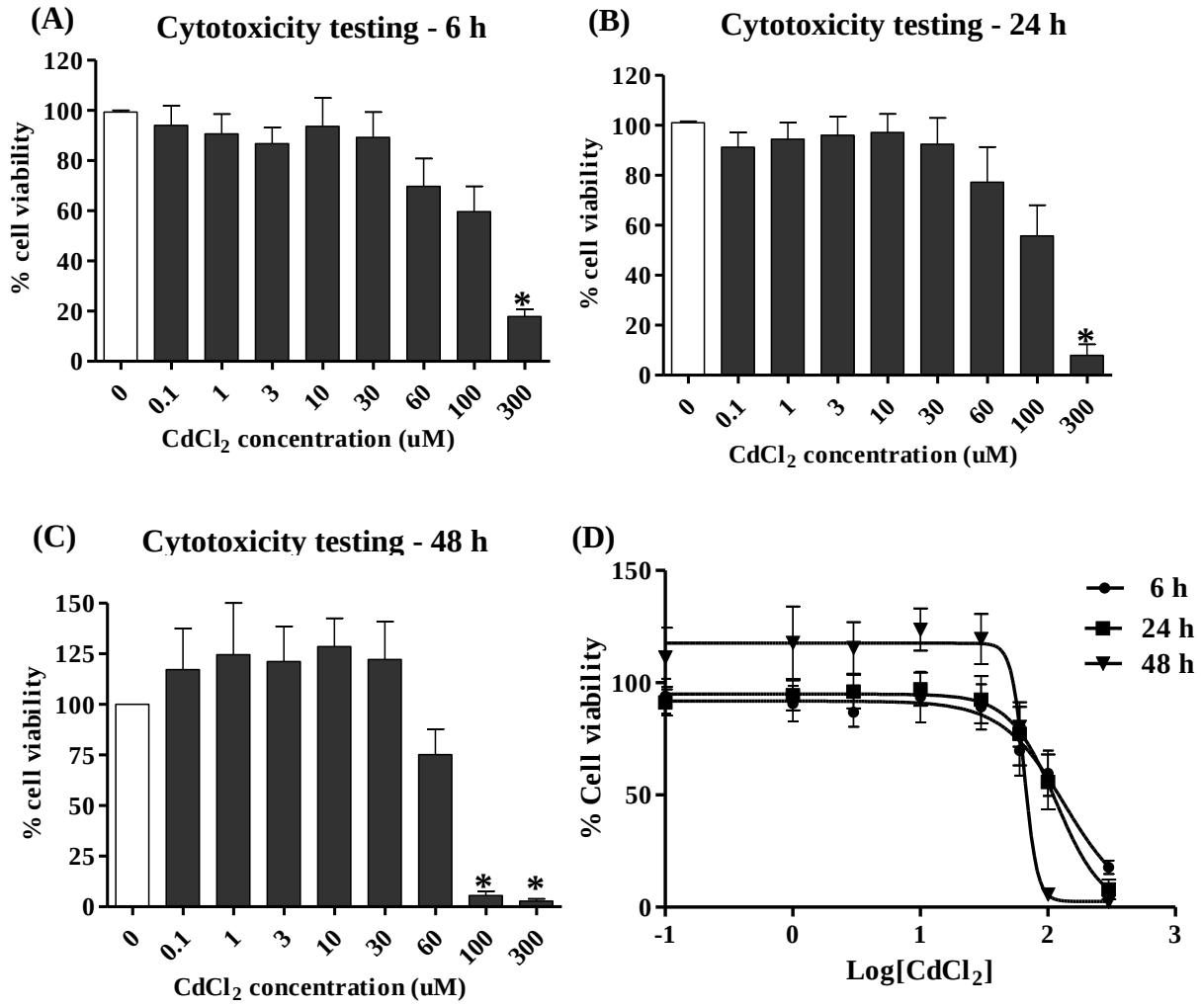

Figure 5.7 Assessment of cadmium toxicity on HCAECs. Cadmium were incubated with HCAECs for 6 (A), 24 (B), and 48 (C) h. (D) Dose–response curve of cell viability following a 6, 24 and 48 h exposure to increasing concentrations of cadmium. Data are means ± SEM. *$P < 0.05$ compared with control (0 µM $CdCl_2$) (n =3, ANOVA with Tukey's multiple test)

5.5 Nitrite levels

5.5.1 Effects of cadmium on NO production in HCAECs

Further, we determined the NO synthesis in endothelial cells by measuring nitrite levels in cells and supernatant from HCAECs using the chemiluminescence method. HCAECs (10^4 cells/cm^2, 800 µL) were seeded into 12 well plates and grown to 80% confluence. The endothelial cells were pre-treated with 0.01 and 0.1 µM of cadmium for 4 h, followed by incubation with 10 µM of acetylcholine for 1 h. Acetylcholine significantly induced NO production by HCAECs cells as indicated by the increase in nitrite levels in cells and supernatants (mean + SEM = 0.27 ± 0.01 and 0.71 ± 0.1 µM, respectively) (Figure 5.8 and 5.9). The acetylcholine-induced NO synthesis in cells was attenuated by 0.1 µM of cadmium. The nitrite levels in cells and supernatants decreased to 0.13 ± 0.01 µM and 0.22 ± 0.12 µM in the presence of acetylcholine.

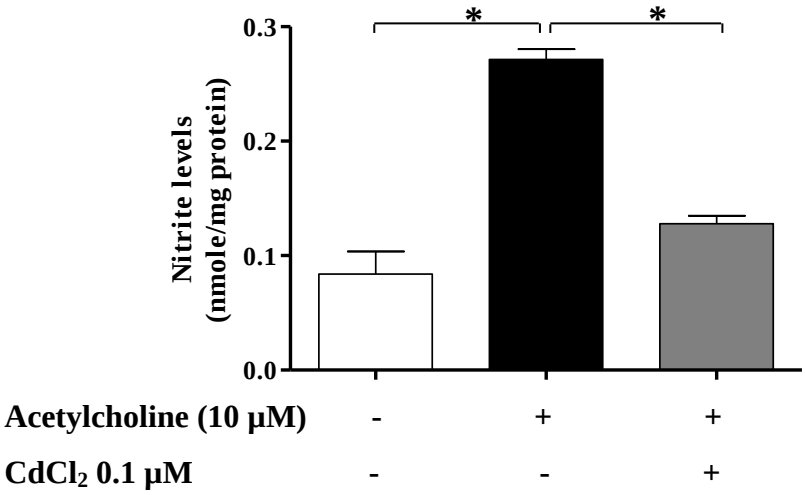

Figure 5.8 Effect of cadmium on nitrite levels in HCAECs. The cells were pre-treated with Cd for 4 h followed by incubation with Ach for 1 h. Nitrite was measured in cell lysate. Data are means ± SEM. *$P < 0.05$ (n = 3, ANOVA with Tukey's multiple test)

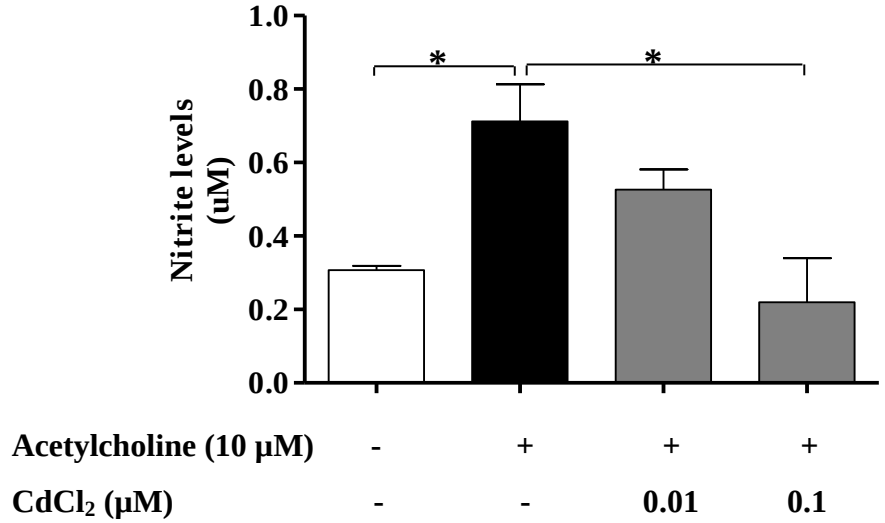

Figure 5.9 Effect of Cd on nitrite levels in supernatant. HCAECs were pre-treated with Cd for 4 h followed by incubation with acetylcholine for 1 h. Data are means + SEM. *P < 0.05 (n = 3, ANOVA with Tukey's multiple test)

5.6 eNOS gene expression

5.6.1 Effects of cadmium on eNOS mRNA levels

In order to examine the effect of cadmium on eNOS transcripts in HCAECs, we performed the quantitative real-time PCR assays. HCAECs (3×10^5 cells/cm^2, 1.5 mL) were seeded into 6-well plates and grown to 80% confluence. The endothelial cells were treated with 0.1, 1 and 10 µM cadmium for 2 h. Cadmium at 0.1, 1 and 10 µM had no effect on the levels of eNOS mRNA when compared with control (Figure 5.10)

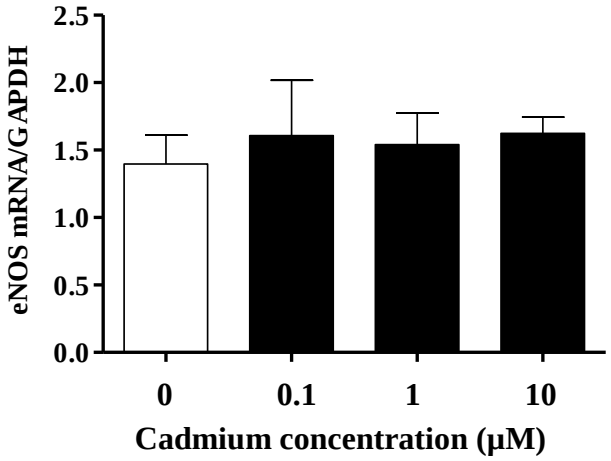

Figure 5.10 Effect of cadmium on eNOS gene expression in HCAECs. The transcript expression was determined by quantitative real-time PCR analysis and normalized to GAPDH mRNA levels. HCAECs were treated with cadmium for 2 h. Data are means ± SEM. (n = 3 each, ANOVA with Tukey's multiple test)

5.7 eNOS protein activity

5.7.1 Effects of cadmium on eNOS protein level and phosphorylation

We further investigated the effects of cadmium on total protein levels and phosphorylation levels of eNOS by immunoblotting techniques. HCAECs (3 x 10^5 cells/cm^2, 1.5 mL) were seeded into 6-well plates and grown to 80% confluence. The endothelial cells were pre-treated with 0.01 and 0.1 µM cadmium for 4 h, and then incubated with 10 µM acetylcholine for 1 h. Cadmium had no effect on eNOS protein levels (Figure 5.11A, middle panel). Acetylcholine-induced eNOS protein phosphorylation at serine 1177 was attenuated by 0.1 µM cadmium (Figure 5.11B). These results suggest that cadmium could inhibit eNOS phosphorylation at serine 1177, leading to a decrease of eNOS activity.

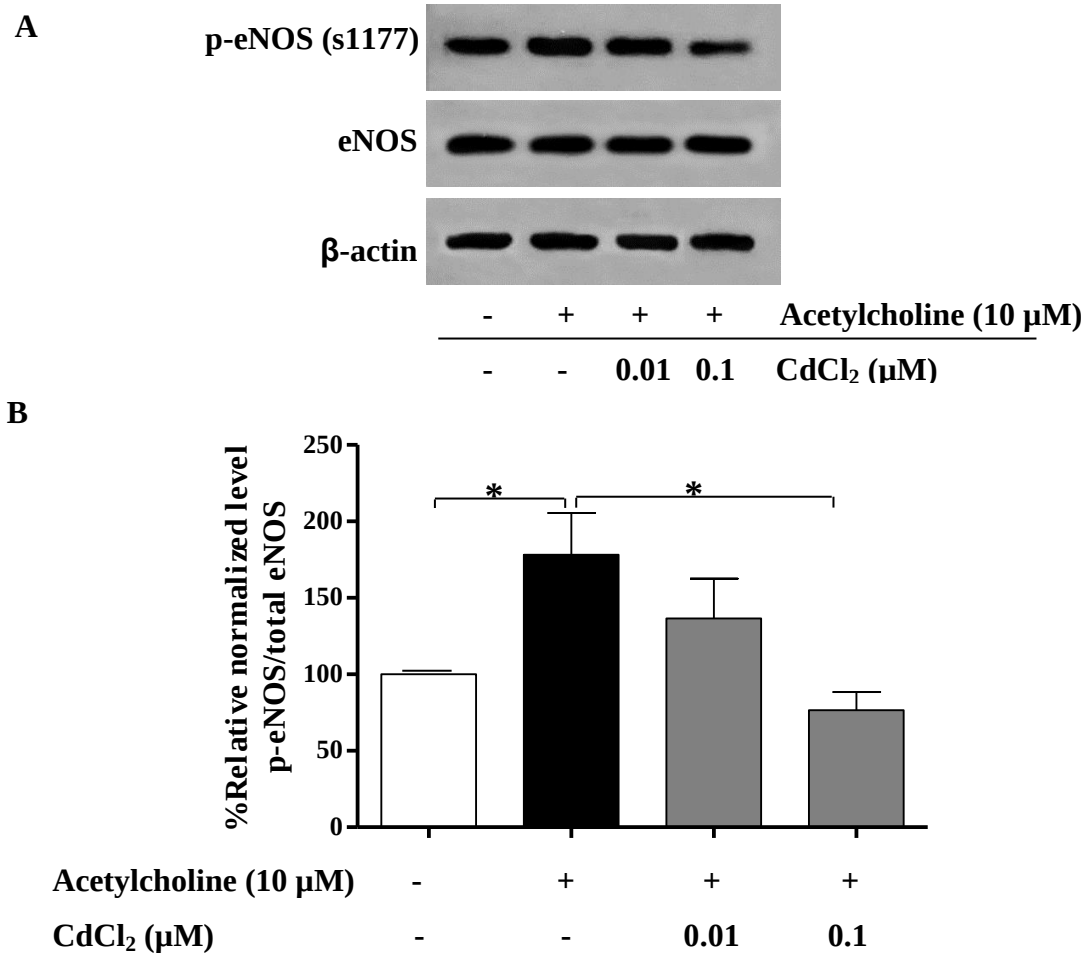

Figure 5.11 Effect of cadmium on total protein levels and phosphorylation levels of eNOS. The endothelial cells were pre-treated with cadmium for 4 h followed by incubating with acetylcholine for 1 h. Phosphorylated eNOS at serine 1177 and total eNOS levels were determined by Western blot analysis. Data are means ± SEM. **P* < *0.05* (n = 3, ANOVA with Tukey's multiple test)