# Iron supplementation protects against lead-induced apoptosis through MAPK pathway in weanling rat cortex

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## Abstract

Recent studies indicate that iron (Fe) is involved in neurotoxicity caused by inorganic lead (Pb). We studied the role of Fe in the effects Pb-induced cerebral apoptosis during rat development and to explore its possible regulatory mechanism. In the present study, weanling male Sprague–Dawley rats were randomly divided into four groups. Three groups of rats received 400 μg/mL Pb acetate solution in drinking water, among which two of the groups were concurrently given 20 mg/kg and 40 mg/kg FeSO$_4$ solution, respectively, as the low and high Fe group, for 6 weeks. The Fe doses were administered orally by gavage every other day according to animal body weight. For the control group, Na acetate with an acetate concentration equivalent to the high dose of Pb acetate was prepared in the same manner. At the end of the study, exposure to Pb in drinking water significantly promoted internucleosomal DNA fragmentation, enhanced the percentage of TUNEL-positive cells and increased the caspase-3 activities in cortex as compared to the controls. At the same time, it did cause a significant decrease in cortex Fe concentrations. Concomitant supplement with different dose Fe appeared to restore brain Fe level to the normal level. Although the low dose of Fe restored brain Pb level to the normal level and the high dose of Fe did not, both of them reduced the formation of DNA fragments, showed few TUNEL-positive cells with yellow nuclei and inhibited Pb-induced procaspase-3 degradation. Western blot showed that exposure to Pb caused a significant elevation in the phosphorylation of ERK1/2, JNK1/2, and Elk-1. Low Fe supplemental treatment suppressed the phosphorylation of ERK1/2 and JNK1/2 but not Elk-1. Interestingly, high Fe treatment slightly suppressed the phosphorylation of JNK1/2, but significantly elevated the phosphorylation of ERK1/2 and Elk-1. Collectively, the current study suggests that supplementation of Fe during Pb treatment prevents against cytotoxicity and apoptosis induced by Pb insults, in which MAPK pathways play an important role in Pb-induced cerebral apoptosis by activating the MEK-ERK pathway that suppresses JNK signaling.

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*Keywords:* Lead; Iron; Apoptosis; ERK; JNK; Cortex

## 1. Introduction

Lead (Pb) is a widespread environmental pollutant and poses a serious health hazard to the developing human brain (Bellinger et al., 1987). Pb is classified as a group 2B carcinogen (possible human carcinogen) by the International Agency for Research on Cancer. Over the past decade, it has become increasingly evident that contact with Pb, even at very low levels, will produce serious adverse health effects, especially in children (Rosen, 1995; Apostoli and Boffetta, 2000). More importantly, Pb is capable of triggering neurological damages and causing severe impairments in neurobehavioral cognitive developments in children (Murata et al., 1993; Sinha et al., 1993). In the central nervous system (CNS), Pb is more readily deposited in the astroglial cells than neurons. Neurons, however, can be damaged indirectly as a result of the response of glial cells to deposited lead (Lindahl et al., 1999). Pb alters the intracellular metal content (Tiffany-Castiglioni et al., 1988) and impairs the differentiation of glial cells, which adversely affects the functions of both glial and neuronal cells (Stark et al., 1992; Kern et al., 1993; Zawia and Harry, 1996).

Iron (Fe) is a critical element for the normal functioning of all cells. Proper levels of Fe are necessary for cells to maintain their viability and proliferation. Cellular Fe deficient with the

iron chelator, deferoxamine (DFO), impairs DNA synthesis in human lymphocytes (Hoffbrand et al., 1976). Reduced DNA synthesis is attributed to a decreased activity of ribonucleotide reductase (Jordan and Reichard, 1998). The ribonucleotide reductase is a Fe-dependent enzyme that converts ribonucleotides into deoxyribonucleotides, which is a prerequisite step for DNA synthesis. As a result, Fe deficiency halts cell cycle progression specifically at the S-phase (Tomoyasu et al., 1993). Fe deficiency also activates the transcription of certain important genes involved in cell cycle control, leading to a cascade of downstream events that contribute to the cell cycle arrest. Enhanced transcription of the tumor suppressor protein, p53, which is directly responsible for the activation of the CIP-1/WAF-1/SDI-1/p21 gene and other p53-responsive genes, has been observed in Fe-deficient cells treated with DFO (Fukuchi et al., 1995) and the subsequent p21 gene product inhibits cyclin-dependent kinases that otherwise would promote cell cycle progression.

Fe accumulation in many organs, on the other had, is correlated with the process of carcinogenesis (Weinberg, 1989). Multiple studies have indicated that cellular Fe efflux could contribute to the prevention and management of cancer (Toyokuni, 1996; Weinberg, 1989). The Fe chelator, DFO, has been shown to induce apoptotic cell death in HL-60 cells (Fukuchi et al., 1994). Other Fe chelators have also been demonstrated to induce apoptotic cell death in various tumors such as human breast cancer cells (T47D-YB and MCF-7) and human neuroblastoma cells (Pahl et al., 2001; Fan et al., 2001).

Apoptosis is a gene regulated phenomenon which is important in both physiological and pathological conditions. The MAPK pathways transduce signals that lead to diverse cellular responses such as cell growth, differentiation, proliferation, apoptosis, and stress responses to environmental stimuli (Geilen et al., 1996; Kortenjann and Shaw, 1995; Marais and Marshall, 1996; Moriguchi et al., 1996; Su and Karin, 1996; Waskiewicz and Cooper, 1995; Xia et al., 1995). The extracellular receptor kinase (ERK) pathway typically transduces growth factor signals that lead to cell differentiation or proliferation (Marais and Marshall, 1996), whereas cytokines and stress signals (e.g., ultraviolet irradiation, heat, synthesis inhibitors) activate the c-Jun NH2-terminal kinase (JNK) and P38 pathways, resulting in stress responses, growth arrest, or apoptosis (Beyaert et al., 1996; Kyriakis and Avruch, 1996; Raingeaud et al., 1995; Waskiewicz and Cooper, 1995; Xia et al., 1995). Signaling through the MAPK pathways culminates in the phosphorylation-dependent activation of a variety of transcription factors that modulate cytokine gene expression (Su and Karin, 1996; Treisman, 1996; Whitmarsh and Davis, 1996).

We hypothesized that, during rat brain development, cellular Fe deficiency contributed to Pb-induced cerebral apoptosis, which may be prevented by Fe supplementation. To test this hypothesis, we exposed the weanling rats to Pb in drinking water and supplemented Fe by oral gavage. We analyzed the brain contents of Pb and Fe by atomic absorption spectrophotometry (AAS) and measured internucleosomal DNA fragmentation and quantified apoptotic cell death. Moreover, we investigated the mechanism of Pb toxicity on cerebral apoptosis by studying the effects of Pb exposure on ERK and JNK MAPKs signaling pathways.

## 2. Materials and methods

### 2.1. Animal exposure

Male Sprague–Dawley rat littermates (Fourth Military Medical University, Xi'an, China) aged 20–22 days and weighing 30–50 g upon arrival were assigned to four groups ($n = 12$) with comparable mean body weights. These animals were housed in stainless-steel cages in a temperature-controlled, 12-h light/12-h dark room with free access to pelleted semi-purified rat chow (Vital Keao Feed Co., Beijing, China) and pre-prepared drinking water. On day 3 (age 22–24 days), the animals started receiving Pb in drinking water and/or Fe by oral gavage. The rat chow is a purified, synthetic diet providing essential nutrients for maintenance, growth, gestation, and lactation of laboratory mice and rats. The ingredient of the diet has been consistent and well controlled with proteins (20.60%), fat (4.16%), fibers (4.92%), and carbohydrates (61%). For essential minerals, the diet contains 1.50% calcium, 0.81% phosphorus, 0.40% potassium, 0.07% magnesium, 0.21% sodium, 0.90 ppm lead, 76 ppm iron, 20 ppm zinc, 65 ppm manganese, 15 ppm copper, 3.2 ppm cobalt, 0.6 ppm iodine, 3.0 ppm chromium, and 0.2 ppm selenium.

The drinking water was prepared by dissolving Pb acetate in distilled, deionized water (Pb acetate 400 μg/mL) and Pb concentrations were verified by a graphite furnace AAS. The Pb exposure paradigm, which was associated with subtle developmental deficits, was chosen according to Cory-Slechta (Cory-Slechta et al., 1983; Zhao et al., 1998; Zheng et al., 1996). Pb concentration in drinking water (400 μg/mL) was chosen because Pb-induced brain hemorrhage was seen at high doses (Holtzman et al., 1984; Press, 1977). Three groups of rats received 400 μg/mL Pb acetate solution in drinking water and two groups were concurrently given 20 mg/kg and 40 mg/kg $FeSO_4$ solution, respectively, as the low and high Fe group, for 6 weeks. The Fe doses were administered orally by gavage every other day according to animal body weight. For the control group, Na acetate with an acetate concentration equivalent to the high dose of Pb acetate was prepared in the same manner.

### 2.2. Collection of tissue samples

At the end of the study, eight animals from each group were decapitated. Brain tissues were excised and weighed. The whole blood samples were used for determination of blood Pb by an electrothermal atomization AAS. Cerebral cortexes were quickly dissected and stored at $-80\,^{\circ}C$ for bioassays described below. Standards for iron (1000 μg Fe/mL) and lead (1000 μg Pb/mL) for AAS were obtained from Alpha Products (Danvers, MA), The brain samples were used for determination of brain Pb by an electrothermal atomization AAS and brain Fe by a flame AAS.

All the procedures involving animal studies were approved by and carried out in accordance with the established guidelines of the local Animal Care and Use Committee.

## 2.3. DNA extraction and electrophoresis

Genomic DNA from brain tissues was purified using a Wizard genomic DNA purification kit (Promega). DNA samples, 10 μg each, were subjected to electrophoresis on a 1.4% agarose at 50 V for 3 h and stained with ethidium bromide and the pattern of DNA cleavage was analyzed as described (Maciejewski et al., 1995).

## 2.4. Quantitation assay of apoptosis

TUNEL assay was performed according to the manufacturer's instructions by using FragEL™ DNA Fragmentation Detection Kit from Promega (Madison, WI). Cortex tissues were fixed in 10% formalin, dehydrated, embedded in paraffin, and cut into sections of 5 μm in thickness. Paraffin sections were dewaxed and dehydrated using xylene and graded alcohol series. The cortical sections were stained using the terminal deoxynucleotidyl transferase (TdT) method. Endogenous peroxidase was first quenched with 2% hydrogen peroxide and the sections were permeabilized using the supplied equilibration buffer. The $3'OH$ ends of DNAs were reacted with TdT and digoxygenin-labeled ATP for 30 min. After washing with PBS, the cells on slides were incubated with an anti-digoxygenin mAb conjugated to peroxidase, washed, and developed with 3,3'-diaminobenzidine (DAB) tetrahydrochloride. Apoptotic cells (TUNEL-positive) were identified by DAB stain and counted at a magnification of ×100. The percentage of apoptotic cells were calculated by dividing the number of TUNEL-positive cells by the total number of cells under a defined area ($10 \, mm^2$).

## 2.5. Western blot analysis

For immunoblotting studies, anti-procaspase-3, anti-ERK1/2 antibody, anti-JNK1/2 antibody, anti-phospho-ERK1/2 antibody, anti-phospho-JNK1/2 antibody and anti-phospho-Elk-1 antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cerebral cortexes were homogenized in RIPA buffer consisting of 50 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.1% SDS, 1% NP-40, 1% deoxycholate, 1% Triton X-100, 10 mM PMSF and 0.1% protease inhibitors cocktail (Roche, Switzerland). After centrifugation at $12,000 \times g$ for 15 min at $4\,°C$, the supernatant was collected and the protein concentration was assayed using Bradford method (Bradford, 1976). The samples were resolved on 12% sodium dodecly sulphate (SDS)-polyacrylamide gel and the proteins in gels were transferred onto nitrocellulose membrane and immunoreactive proteins were visualized using West Pico Chemiluminescent kit (Pierce, Rockford, IL, USA) and the density of protein bands quantified by transmittance densitometry using volume integration with LumiAnalyst Image Analysis software.

## 2.6. Caspase-3 activity assay

The activity of caspase-3 was determined using the Caspase-3 activity kit (Beyotime Institute of Biotechnology, Haimen, China). To evaluate the activity of caspase-3, cerebral cortexes were homogenized in 100 μL reaction buffer (1% NP-40, 20 mM Tris–HCl (pH 7.5), 137 mM Nad and 10% glycerol) containing 10 μL caspase-3 substrate (Ac-DEVD-pNA) (2 mM). Lysates were incubated at $37\,°C$ for 2 h. Samples were measured with an ELISA reader at an absorbance of 405 nm.

## 2.7. Statistical analysis

The results were expressed as mean ± S.D. Difference between means was determined by one-way ANOVA followed by a Student–Newman–Keuls test for multiple comparisons. A probability value of $p < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Blood Pb level following Pb exposure

Chronic exposure to Pb in drinking water under the current dose regimen resulted in a 6.6-fold increase in blood Pb (BPb) as compared to control rats ($p < 0.05$) (Fig. 1). Concomitant supplement with low-dose Fe (7 mg Fe/kg) by oral gavage significantly reduced BPb in Pb-exposed rats; but it did not completely restore BPb to the normal level seen in control rats (Fig. 1). Interestingly, the high dose of Fe (14 mg Fe/kg) did not reduce, but instead increased BPb in Pb-exposed rats (Fig. 1).

### 3.2. Pb treatment induces cell apoptosis in cortex

In this study, we subjected weanling rats to Pb or Pb supplemented with Fe. Analysis of genomic DNA from the brain tissues of weanling rats treated with Pb by agarose electrophoresis revealed internucleosomal DNA fragmentation as evidenced
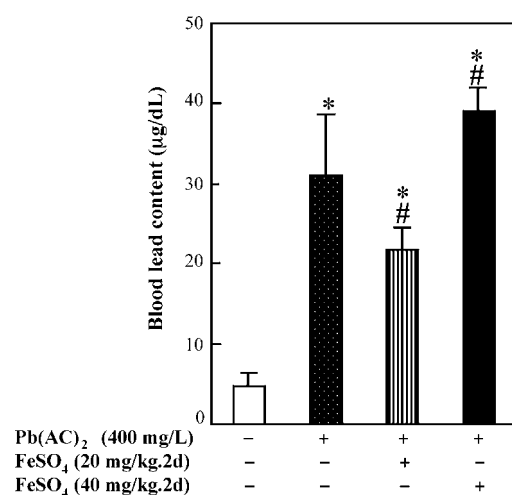


Fig. 1. Pb concentrations in blood following Pb exposure and concomitant Fe supplement treatment. Weanling male Sprague–Dawley rats were exposed to Pb as 400 μg/mL Pb acetate in drinking water daily and concomitantly administered orally by gavage once every other day with 20 mg/kg $FeSO_4$ solution (as the low Fe supplement group) or 40 mg/kg $FeSO_4$ (as the high Fe supplement group), for 6 weeks. Data represent mean ± S.D. ($n = 8$), $^*p < 0.05$ compared to controls; $^\#p < 0.05$ compared to Pb-alone group.

by the laddering patterns of DNA characteristic of apoptosis (Fig. 2A and B). Cortical DNA from control rats, however, yielded intact DNA. To further confirm the occurrence of apoptosis, TUNEL assay was used to detect the presence of DNA strand breaks. Fig. 2 showed *in situ* TUNEL staining (Fig. 3A) and the percentage (%) of apoptotic cells (Fig. 3B) in the cerebral cortex of control and Pb-treated animals. Control rats showed

few TUNEL-positive cells with yellow nuclei (Fig. 3A and B). In Pb-treated rats, Pb-induced apoptosis was obviously seen because of 51% of TUNEL-positive cells (Fig. 3A and B). We also examined the activation of caspase-3 which had been shown to play a pivotal role in the execution phase of apoptosis induced by diverse stimuli (Lockshin, 2005). Accordingly, Pb exposure declined the levels of procaspase-3 (Fig. 4) but elevated activation of caspase-3 (Fig. 5).

### 3.3. Pb-induced apoptosis is attributed to both increased Pb level and decreased Fe level in cortex

Recent studies have demonstrated that administration of Pb can reduce the absorption of Fe in brain. To investigate whether Pb-induced apoptosis is related to cerebral Fe status, we examined cortex Pb and Fe levels by AAS. Chronic exposure to Pb in drinking water under the current dose regimen resulted in about 1.54-fold increase in cortex Pb concentration (Fig. 6A). There was also a concurrent significant decrease in brain Fe concentration in the cortex (Fig. 6B). Results suggest that Pb exposure induces a disturbance of cellular Fe homeostasis in the cerebral cortex, which may be the major reason of Pb-induced cerebral apoptosis.

### 3.4. Iron supplementation during Pb treatment suppresses Pb-induced cerebral cell apoptosis

To further investigate the role of Fe in Pb-induced cerebral cell apoptosis, we supplemented various doses of Fe to rats receiving Pb and examined by AAS the Pb and Fe levels in the cortex of rats receiving different doses of Fe supplementation. The low-dose Fe supplement significantly reduced Pb levels in the cortex compared to the Pb-only group (Fig. 6A). Cortex Fe levels after low-dose Fe treatment were not significantly different from those in control rats (Fig. 6B). The agarose gel electrophoresis analysis showed significantly reduced amounts of DNA fragments (Fig. 2B). Accordingly, the low-dose Fe supplemented rats showed fewer TUNEL-positive cells with yellow nuclei (Fig. 3) and lower procaspase-3 degradation (Figs. 4 and 5). The high-dose Fe supplement did not decrease brain Pb levels (Fig. 6A), but reinstated the brain Fe to the normal levels seen in control rats (Fig. 6B). Supplementation of high-dose Fe during Pb treatment also reduced the formation of DNA fragments (Fig. 2B), decreased 22% of TUNEL-positive apoptotic cells (Fig. 3) and inhibited Pb-induced procaspase-3 degradation (Figs. 4 and 5), suggesting that Fe supplementation attenuated Pb-induced apoptosis in the cerebral cortex. These results supported the hypothesis that supplementation of Fe during Pb treatment inhibited Pb-induced cerebral cell apoptosis, which is at least in part responsible for brain Fe homeostasis.

### 3.5. Pb-induced cell apoptosis is elevated by decreased Fe through JNK MAPK pathway

Pb-induced cerebral cell apoptosis could be due to the effect of Pb on MAPK pathway, which is involved in cell growth,
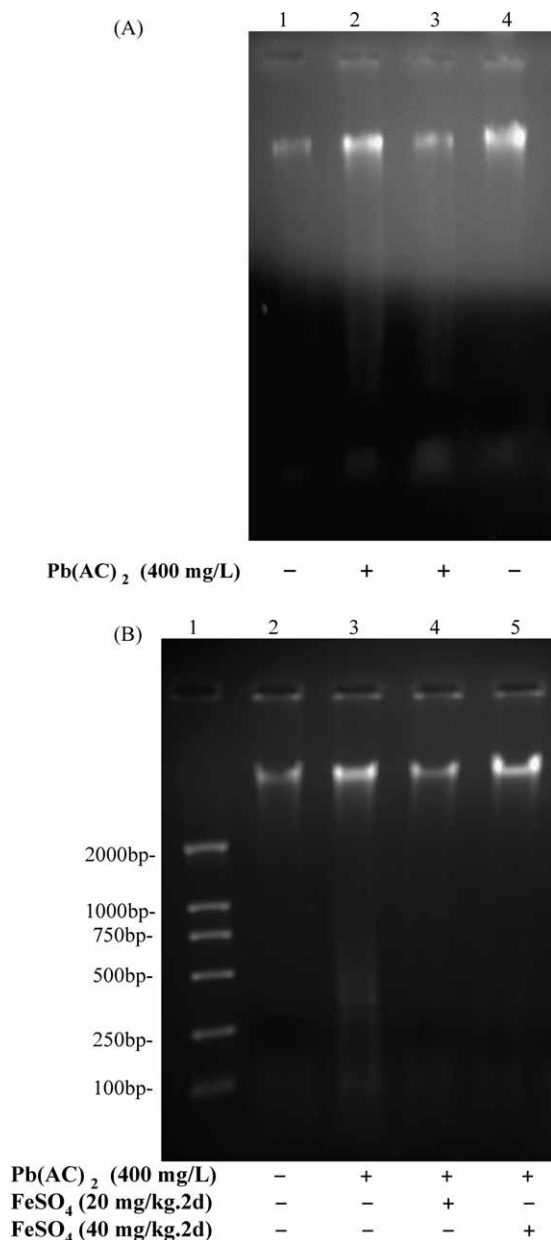


Fig. 2. Agarose gel electrophoretic pattern of cortical DNA. (A) Weanling male Sprague–Dawley rats were exposed to Pb in drinking water daily for 6 weeks. Lane 1: control rat cortex; lane 2: cortex of Pb-exposed rat; lane 3: cortex of Pb-exposed rat; lane 4: control rat cortex. (B) Weanling male Sprague–Dawley rats were exposed to Pb in drinking water daily and given Fe(II) by oral gavage every other day, for 6 weeks. Lane 1: marker; lane 2: control rat cortex; lane 3: cortex of Pb-exposed rat; lane 4: cortex of Pb-exposed rat concomitantly treated with low-dose Fe; lane 5: cortex of Pb-exposed rat concomitantly treated with high dose Fe. DNA samples were electrophoresed on 1.4% agarose gel, stained with ethidium bromide and visualized under UV-transilluminator. The results are representative four independent experiments.
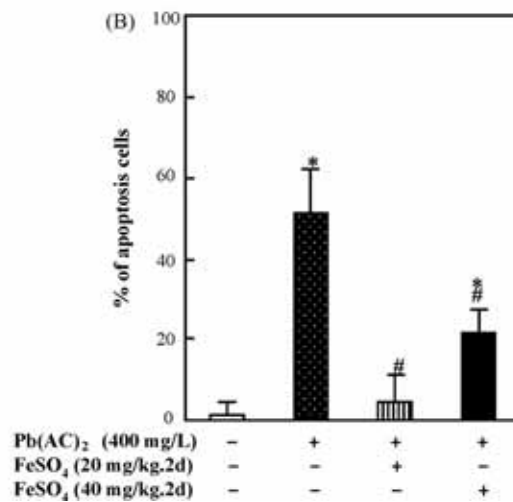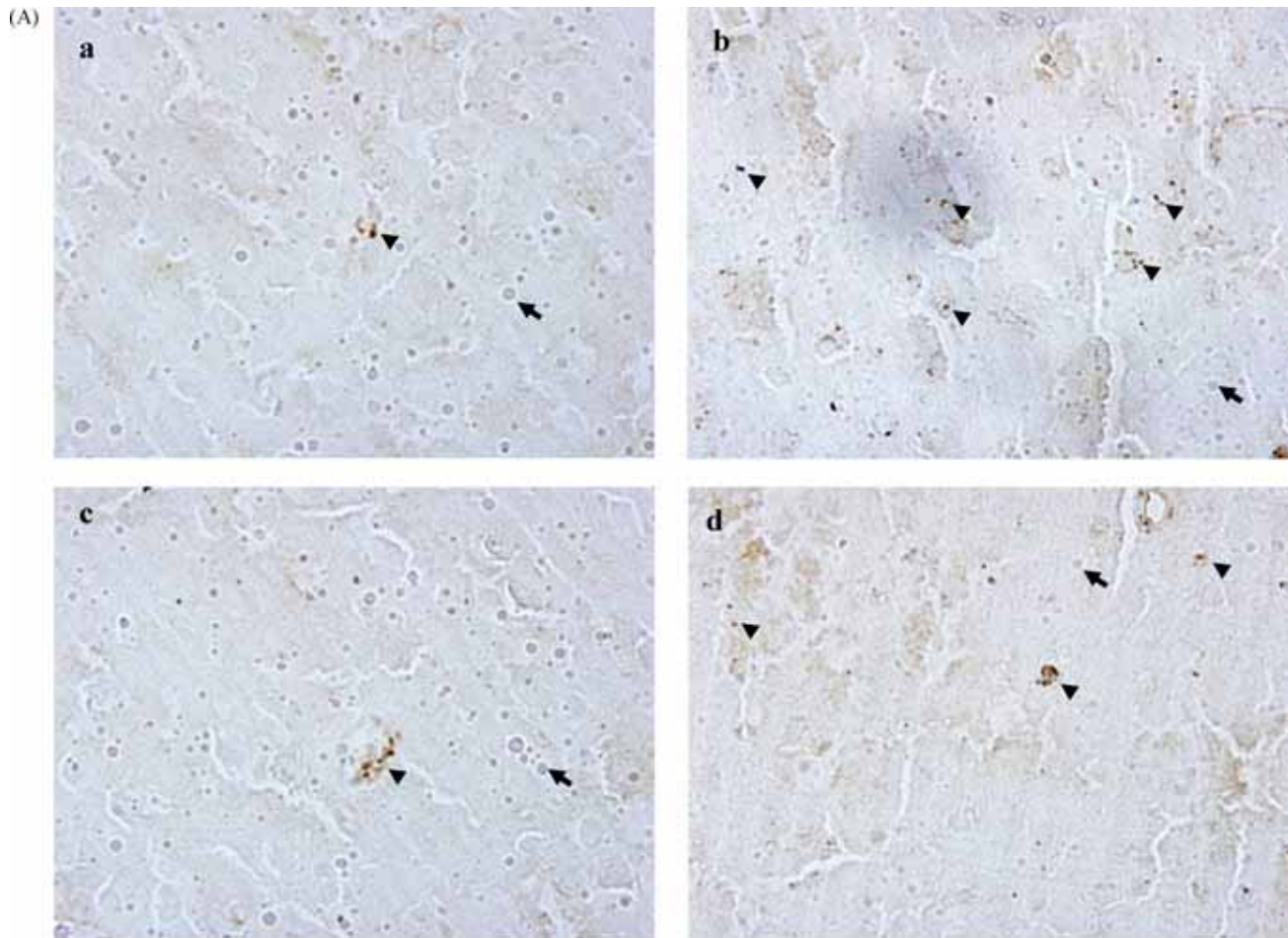
Fig. 3. *In situ* TUNEL staining (A) and percentage of apoptotic cells (B) in the cerebral cortex of control and experimental animals. Apoptosis was detected as detailed in experimental procedures. Apoptotic cells were identified by DAB stain (magnification, ×100). Percentage of apoptotic cells is calculated from apoptotic (arrowhead) and non-apoptotic (arrow) cells. (A) The results are representative four independent experiments. (a) Control rat cortex; (b) cortex of Pb-exposed rat; (c) cortex of Pb-exposed rat concomitantly treated with low-dose Fe; (d) cortex of Pb-exposed rat concomitantly treated with high dose Fe. (B) Results are expressed as mean ± S.D. ($n = 3$), $^*p < 0.05$ compared to controls; $^\#p < 0.05$ compared to Pb alone group.

different and apoptosis. To test whether increased cell apoptosis from decreased Fe and increased Pb during Pb treatment is related with MAPK pathway, we examined the phosphorylation status of JNK1/2 in cortex by immunoblotting studies.

Exposure of young animals to Pb led to a pronounced increase in the expression of phosphorylated JNK1/2 as compared to the controls (Fig. 7A and B). Low-dose Fe supplementation among these rats restored the normal expression of phosphorylated
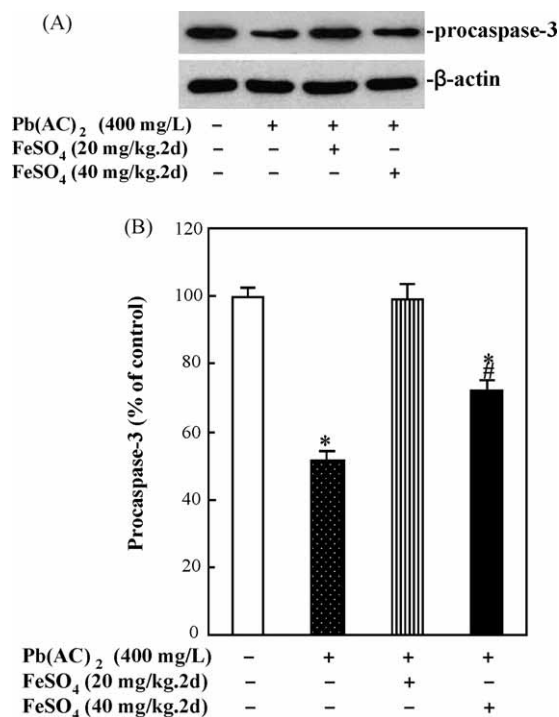
Fig. 4. Activation of procaspase-3 in rat cortex following Pb exposure and concomitant Fe supplement treatment. Animal treatment has been described in Fig. 1. Cerebral cortexes from each group were prepared for Western blot. (A) Representative blots of the activation of procaspase-3 in cerebral cortex. (B) The density ratios of the activation of procaspase-3 were first calculated. The changes were expressed as the percentage of controls. Data represent mean ± S.D. ($n = 3$), $^{*}p < 0.05$ compared to controls; $^{\#}p < 0.05$ compared to Pb alone group.

JNK1/2 (Fig. 7A and B). Interestingly, the phosphorylation of JNK1/2 in high-dose Fe rats was reduced as compared to the only Pb-treated, but still more than the control, which agrees with the results of the apoptosis assay (Figs. 3–5). These
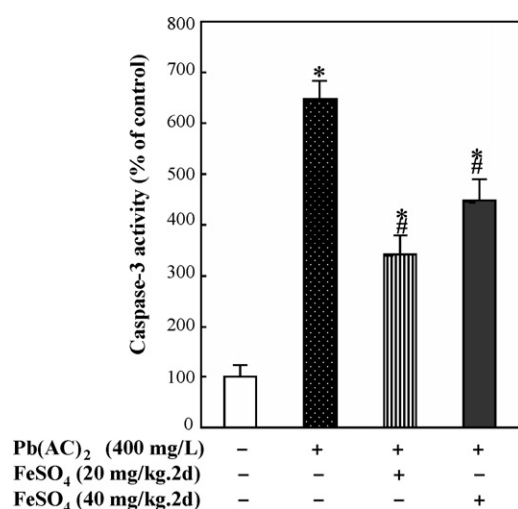


Fig. 5. Caspase-3 activity in rat cortex following Pb exposure and concomitant Fe supplement treatment. Animal treatment has been described in Fig. 1. The changes were expressed as the percentage of controls. Data represent mean ± S.D. ($n = 8$), $^{*}p < 0.05$ compared to controls; $^{\#}p < 0.05$ compared to Pb alone group.
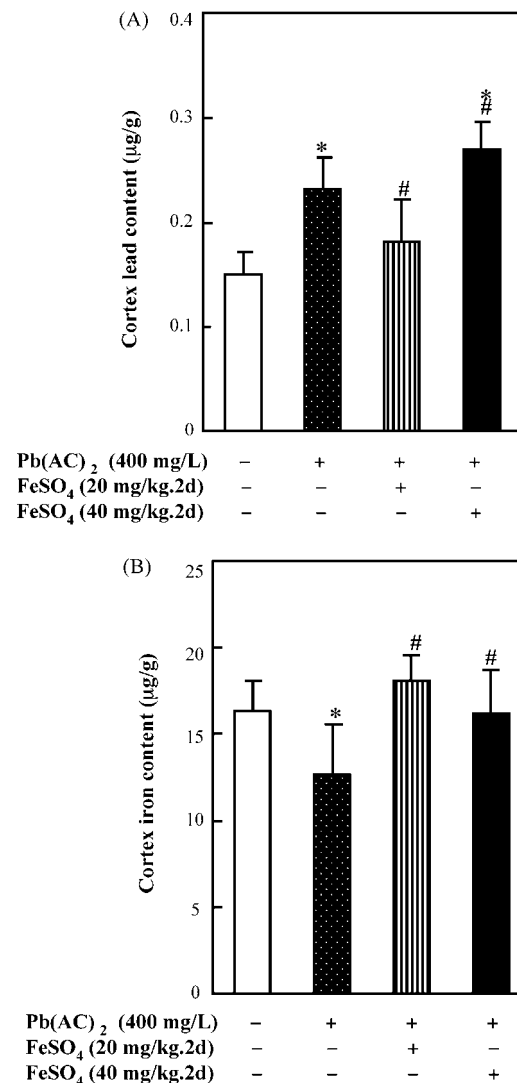


Fig. 6. Pb (A) and Fe concentrations (B) in rat cortex following Pb exposure and concomitant Fe supplement treatment. Animal treatment has been described in the legend to Fig. 1. Data represent mean ± S.D. ($n = 8$), $^{*}p < 0.05$ compared to controls; $^{\#}p < 0.05$ compared to Pb alone group.

findings imply that JNK MAPK pathway affected by Fe is an important regulatory event in Pb-induced cerebral apoptosis.

### 3.6. Fe supplementation through activation of the MEK-ERK pathway abolishes Pb-induced cerebral apoptosis

To study the role of the MEK-ERK pathway in Pb-induced apoptosis, we also investigated the levels of phosphorylated and total ERK. Pb treatment caused a slight increase in ERK activity (138–140%; Fig. 8A and B). This activation of ERK was suppressed after supplementation of low-dose Fe, and was almost reduced to the basal level (120–121%). Interestingly, supplementation of high dose Fe significantly promoted the activity of ERK which was more than the level of treatment with Pb (155–162%). These findings support the notion that activation of ERK in Pb-induced cerebral apoptosis delivers a survival signal that counteracts the proapoptotic effects associated with the activation of JNK MAPK.
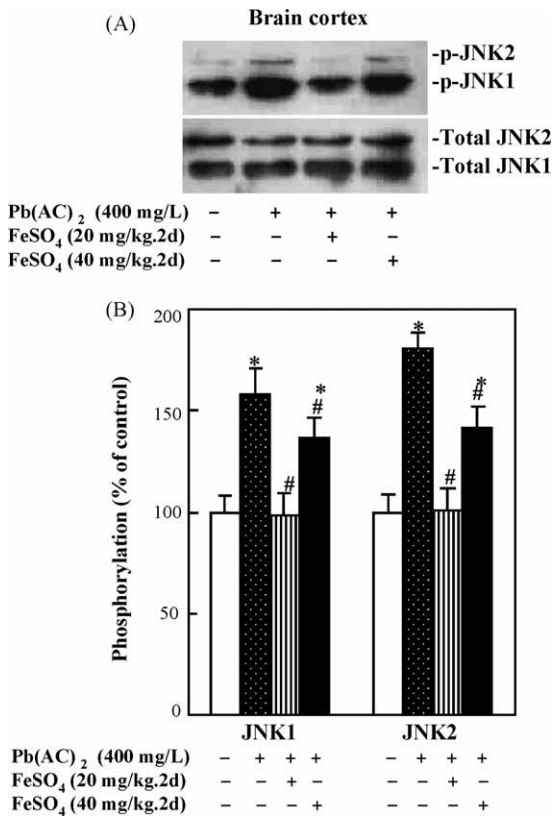
Fig. 7. Western blot analysis of the phosphorylation of JNK1/2 in rat cortex. Animal treatment has been described in Fig. 1. Cerebral cortexes from each group were prepared for Western blot. (A) Representative blots of the phosphorylation of JNK1/2 in cerebral cortex. (B) The density ratios of the phosphorylation of JNK1/2 were first calculated. The changes were expressed as the percentage of controls. Data represent mean ± S.D. ($n = 3$), $^*p < 0.05$ compared to controls; $^#p < 0.05$ compared to Pb alone group.



Fig. 8. Western blot analysis of the phosphorylation of ERK1/2 in rat cortex. Animal treatment has been described in Fig. 1. Cerebral cortexes from each group were prepared for Western blot. (A) Representative blots of the phosphorylation of ERK1/2 in cerebral cortex. (B) The density ratios of the phosphorylation of ERK1/2 were first calculated. The changes were expressed as the percentage of controls. Data represent mean ± S.D. ($n = 3$), $^*p < 0.05$ compared to controls; $^#p < 0.05$ compared to Pb alone group.

## 3.7. Effects of Fe supplementation on the phosphorylation of Elk-1 in cortex

Elk-1 can be phosphorylated and activated by ERK and JNK MAPKs, resulting in enhanced SRE-dependent c-Fos expression (Karin, 1995; Whitmarsh and Davis, 1996). By Western blotting, we found that Pb exposure strongly elevated the phosphorylation of Elk-1 (approximately four times higher than levels observed in untreated rats; Fig. 9A and B). The phosphorylation of Elk-1 was also activated by different doses of Fe supplementation (Fig. 9A and B). Supplementation of low-dose Fe had the strongest activation, and, although slightly less than that seen in low-dose Fe supplementation, the phosphorylation of Elk-1 by high dose Fe was still significantly increased relative to only Pb-treated group (Fig. 9A and B).

## 4. Discussion

In the present investigation, we observed that exposure to Pb in drinking water during development caused MAPK-dependent cerebral cell apoptosis in cortex, which involved phosphorylation and degradation of ERK and JNK. The most interesting finding of this study was that Pb-induced apoptosis may, at least in part, be due to a decrease of brain Fe content.
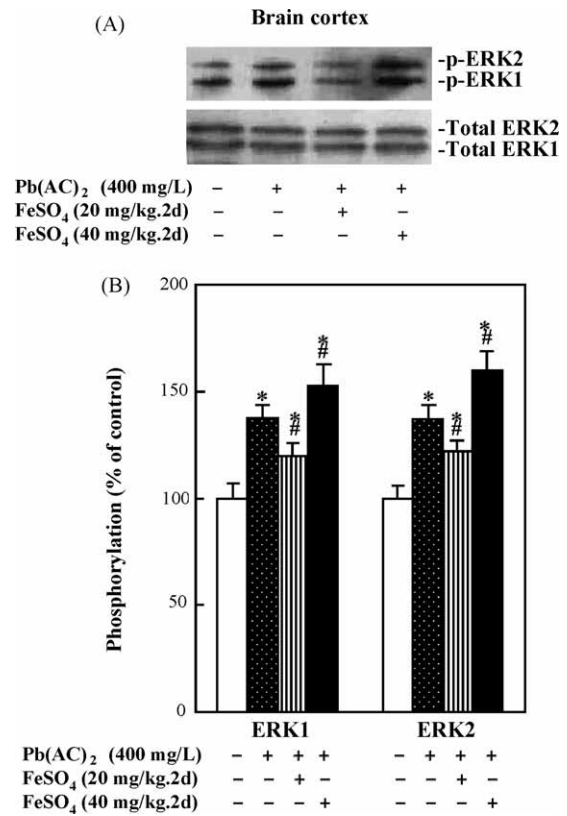
Several studies demonstrated that Pb produced neuronal apoptosis in whole animals and cultured cells. Low to moderate level Pb exposure produces apoptotic rod and bipolar cell death in developing and adult rats (Fox and Chu, 1988; Fox et al., 1997) and apoptotic neuronal cell death in primary cultured cells (Oberto et al., 1996; Scortegagna and Hanbauer, 1997). Consequently, alterations in neural circuitry may underlie some of the neurological effects of Pb exposure during brain development. At present, the molecular mechanism of Pb-induced apoptosis remains unclear. In this study, we confirmed that Pb-induced toxicity in cerebral cortex was associated with apoptosis. Interestingly, we found that Pb-induced apoptosis in cortex was related with Fe level by examination of brain Pb and Fe levels. To further confirm the involvement of Fe in apoptosis, we supplemented Fe to restore Fe level to normal, and then examined their state of apoptosis. Results showed that supplementation of Fe during Pb treatment reduced the formation of DNA fragments and decreased the percentage of TUNEL-positive apoptotic cells (Fig. 3) and inhibited Pb-induced procaspase-3 degradation (Figs. 4 and 5), which shows the potential prevention of Pb-induced apoptosis.

It should be noted that the high dose of Fe supplement (14 mg Fe/kg) did not lower blood Pb, nor did it reduce cortex Pb concentrations, but it did protect Pb-induced cellular
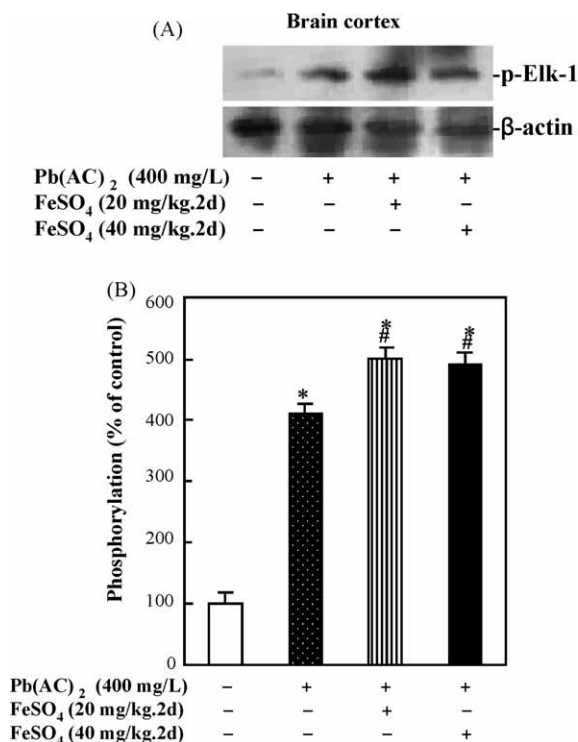
(A)
**Brain cortex**

-p-Elk-1

-β-actin

Pb(AC)$_2$ (400 mg/L)    −    +    +    +
FeSO$_4$ (20 mg/kg.2d)    −    −    +    −
FeSO$_4$ (40 mg/kg.2d)    −    −    −    +



Fig. 9. Western blot analysis of the phosphorylation of Elk-1 in rat cortex. Animal treatment has been described in Fig. 1. Cerebral cortexes from each group were prepared for Western blot. (A) Representative blots of the phosphorylation of Elk-1 in cerebral cortex. (B) The density ratios of the phosphorylation of Elk-1 were first calculated. The changes were expressed as the percentage of controls. Data represent mean ± S.D. ($n = 3$), $^*p < 0.05$ compared to controls; $^{\#}p < 0.05$ compared to Pb alone group.

apoptosis. The less prevention of it could be due to Pb itself neurotoxicity in cell apoptosis, such as direct Pb oxidative effects (Adonaylo and Oteiza, 1999).

At present, a great part of the damage caused by Pb in cellular physiology is caused by its ability to substitute for diverse polyvalent cations (calcium, zinc, magnesium and other divalent cation) in their binding sites (Godwin, 2001). These interactions allow Pb to affect different biologically significant processes, including metal transport, energy metabolism, apoptosis, ionic conduction, cell adhesion, inter- and intracellular signaling, diverse enzymatic processes, protein maturation, and genetic regulation. The effect of cellular Fe deficiency can be attributed to decreased activity of ribonucleotide reductase (Jordan and Reichard, 1998), a Fe-dependent enzyme that converts ribonucleotides into deoxyribonucleotides as a prerequisite step for DNA synthesis. As a result, there is growth arrest of Fe-deficient cells in the S-phase of the cell cycle (Tomoyasu et al., 1993). Our results suggested that Pb treatment disrupted cellular Fe homeostasis, which partly contributed to Pb-induced apoptosis. At the cell membrane, Pb produces peroxidative damage to lipids and proteins. This effect seems to be caused by a combination of such mechanisms as Fe release (which in turn is involved in free radical formation), disruption of antioxidant mechanisms, and direct Pb oxidative effects (Villeda-Hernandez et al., 2001; Adonaylo and Oteiza, 1999).

To gain further insight into the mechanism of Pb-induced cerebral apoptosis, we examined the possible involvement of members of the MAP kinase subfamily, because increasing evidences suggest the important regulatory roles of MAP kinases in many different physiological and pathological settings (Ip and Davis, 1998). Within cells, there are positive and negative regulatory pathways of apoptosis, and it is the balance between these pathways that determines cell fate. Depending upon the cell context, SAPK/JNK activation can lead to proliferation, differentiation or apoptosis (Whitmarsh and Davis, 1996; Paul et al., 1997). The JNK pathway is implicated in inhibiting bcl-2, which is an anti-apoptotic factor, and phosphorylation of an essential transcription factor, c-Jun (Matsuzawa and Ichijo, 2001). In addition, ERK is shown to have anti-apoptotic effects (Yoshino et al., 2001; Nebreda and Gavin, 1999). For Pb, there are only few studies on its effects on MAPKs, or their downstream effectors. However, stimulation of ERK, JNK, and p38 MAPK phosphorylation by Pb has been demonstrated in cell cultures (Leal et al., 2002; Ramesh et al., 1999; Williams et al., 2000). In the present study, we investigated the ability of lead to activate the MAPK cascade. Our results showed that JNK MAPK enhanced Pb-induced cerebral apoptosis through counteraction of MER-ERK pathway. ERK1 and ERK2 (p42 and p44 MAPK) are the best studied MAPK, which are activated by mitogens and play a central role in cell proliferation. Therefore, it is possible that disruption in the regulation of ERK pathway could participate in the molecular mechanisms of Pb neurotoxicity. By Western blot analysis, we found that Fe supplementation through activation of the MEK-ERK pathway abolished Pb-induced cerebral apoptosis. Suppressed JNK signaling pathway attenuated Pb-induced cerebral apoptosis by regulation of transcription factor such as Elk-1. More recently, studies have shown that JNK triggers the mitochondrial apoptosis pathway via activation of Bax and Bim after cerebral ischemia (Okuno et al., 2004; Gao et al., 2005).

In conclusion, we have shown here that Pb-induced cell death in cortex in vivo may partly be due to apoptosis and that apoptosis phenomenon may partly be due to the actual decrease of cortex Fe. Moreover, we found that Fe supplement protects against cytotoxicity and apoptosis induced by Pb insults, in which MAPK pathways play an important role in Pb-induced cerebral apoptosis by activating the MEK-ERK pathway that suppresses JNK signaling. These observations assist our understanding of the mechanism of Pb toxicity in children for better design of therapeutic strategy for children with lead poisoning.

## Acknowledgements

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