BIOMEDICAL AND ENVIRONMENTAL SCIENCES **19**, 15-20 (2006)

# Fenvalerate-induced Alterations in Calcium Homeostasis in Rat Ovary[1]

JUN HE, JIAN-FENG CHEN, RU LIU, LIN SONG, HEBRON C. CHANG, AND XIN-RU WANG[2]

*Jiangsu Key Laboratory of Applied Toxicology, Nanjing Medical University, Nanjing 210029, Jiangsu, China*

**Objective** To observe the effects of fenvalerate on calcium homeostasis in rat ovary. **Methods** Female Sprague-Dawley rats were orally given fenvalerate at daily doses of 0.00, 1.91, 9.55, and 31.80 mg/kg for four weeks. The ovary ultrastucture was observed by electron microscopy. Serum free calcium concentration was measured by atomic absorption spectrophotometry. The activities of phosphorylase *a* in rat ovary were evaluated by the chromatometry. The total content of calmodulin in ovary was estimated by ELISA at each stage of estrous cycle. Radioimmunoassay (RIA) was used to evaluate the level of serum progesterone. **Results** Histopathologically, damages of ovarian corpus luteum cells were observed. An increase in serum free calcium concentration was observed in rats treated with 31.80mg/kg fenvalerate. The activities of phosphorylase *a* enhanced in all treated groups, and fenvalerate increased the total content of calmodulin significantly in estrus period. Serum progesterone levels declined in fenvalerate exposed rats in diestrus. **Conclusion** Fenvalerate interferes with calcium homeostasis in rat ovary. Also, the inhibitory effects of fenvalerate on serum progesterone levels may be mediated partly through calcium signals.

**Key words**: Pyrethroids; Fenvalerate; Rat ovary; Calcium homeostasis; Progesterone

## INTRODUCTION

Fenvalerate[*4-Chloro-α-(1-methylethyl)benzenea ceticacid cyano(3-phenoxyphenyl) methyl ester*], a member of the family of synthetic pyrethroid, type Ⅱ, is commonly used as a highly active insecticide in agricultural and other domestic applications in China. It is generally believed that fenvalerate is less harmful to the environment than most other insecticides. Although fenvalerate is relatively immobile and readily degraded in soil, it is moderately persistent in soils because of low water solubility and high octanol-water partition coefficients[1]. Also, fenvalerate has been identified as having the potential to accumulate in aquatic sediments and biota. Therefore, with increased application, it is necessary to explore the possible toxicity of fenvalerate comprehensively. Fenvalerate has been found to cause severe neurotoxic effects. Recently, fenvalerate has been linked to endocrine disruption. It is enlisted as one of the endocrine-disrupting chemicals (EDCs) and has aroused worldwide concern. EDCs are known to act at multiple sites through multiple modes of action, but the mechanisms of action are poorly understood[2]. It is essential to clarify the mechanisms of action from multiple viewpoints.

Calcium functions are a second messenger. A large number of cellular and extracellular events have been identified as being subjected to calcium regulation. Based on the significant role of the $Ca^{2+}$ messenger system in numerous key cell processes, it is logical to examine the possible disturbance in $Ca^{2+}$ homeostasis and $Ca^{2+}$-mediated functions as the underlying mechanisms of toxicity[3]. Several studies suggest that some toxic symptoms induced by pyrethroids are associated with cell calcium homeostasis. The changes induced by fenvalerate in circulatory $T_3$ and $T_4$ are accompanied with increased levels of total calcium as well as protein-bound calcium in whole brain and hypothalamus[4]. Deltamethrin, another type Ⅱ pyrethroid, causes increase in neurotransmitter release and intrasynaptosomal free $Ca^{2+}$ levels and protein phosphorylation activities at the same time[5]. In addition, pyrethroids can bind to the $Ca^{2+}$, $Mg^{2+}$-ATPase adopting a folded conformation with both the acid and alcohol moieties in contact with

[1]This work was supported by National Natural Science Foundation of China (No. C03010501), the Preliminary Study of an Important Project in the National Basic Research (200150) and the Greatest Project in the National Basic Research (2002CB512908).

[2]Correspondence Should be addressed to Xin-Ru WANG.

Biographical note of the first author: Jun HE, born in 1973, female, master, majoring in endocrine and reproductive toxicology.

0895-3988/2006
CN 11-2816/Q

hydrophobic regions of the ATPase[6].

However, to our knowledge, no studies have been carried out to address the effects of pyrethroids on calcium homeostasis in ovary. Alterations in intracellular calcium homeostasis in ovary may lead to dysfunctions of the reproductive and endocrine systems. Furthermore, accumulating evidence indicates that calcium ions modulate gonadotropin-stimulated steroidogenesis by granulosa cells[7-8]. The calcium-calmodulin system participates in the regulation of steroidogenesis at different stages of granulosa cell differentiation[9].

In the present work, we investigated the effects of fenvalerate on calcium homeostasis in ovaries in female SD rats exposed to fenvalerate for four weeks. Then, the serum progesterone (P4) concentrations after fenvalerate exposure were measured.

## MATERIALS AND METHODS

### *Chemicals*

Fenvalerate (>85% purity) was obtained from Jiangsu Hormone Institute (China). Glycylglycine and glucose 1-phophate were purchased from Sigma Chemical Co. (St, Louis, MO). Recombinant human calmodulin (CaM) was purchased from Nanjing General Hospital of Nanjing Military Command (Jiangsu, China). The CaM polyclonal antibody originated from rabbits was purchased from Santa Cruz (CA, USA). All the other chemicals used in the present study were of reagent grade.

### *Animals and Treatments*

Female Sprague-Dawley rats were purchased from Animal Center of Chinese Academy of Sciences (Shanghai, China). The mean weight on arrival was $180 \pm 10$ g ($\bar{x} \pm s$). The rats were kept in central animal facilities (room temperature, 20℃-22℃; relative humidity, 50%-60%) away from any known contaminants in a 12 h dark-light cycle. The animals had free access to food and water and the base diet was soybean free.

The $LD_{50}$ of fenvalerate in experimental rats was 477.5 mg/kg determined by Karber's method.

During the study period, daily vaginal smears were taken between 14:00 pm and 15:00 pm to monitor the estrous cycle. Estrous stages were classified as proestrus (P), estrus (E), metaestrus (M) and diestrus (D)[10]. All the vaginal smear slides were observed by one individual.

All animals were divided into one control and three treated groups ($n$=25 per group). The fenvalerate in corn oil was orally given at daily doses of 31.8 mg/kg (1/15 $LD_{50}$), 9.55 mg/kg (1/50 $LD_{50}$) and 1.91 mg/kg (1/250 $LD_{50}$) respectively for 4 weeks (five days a week). The same volume of corn oil was given to control animals. At the end of study, the animals were sacrificed by decapitation. Blood was collected and serum was frozen at -20℃ while the ovaries were removed and stored at -80℃ till analysis.

### *Ovary Histopathology Study by Electron Microscopy*

The removed ovaries were selected randomly and fixed in 5% glutaraldehyde immediately. After being washed 4 times in PBS, the samples were stained with uranyl acetate and processed through standard dehydration in graded ethanol before infiltration and embedded in epoxy resin. The ultrathin sections were obtained with an ultramicrotom , stained with uranyl acetate and lead citrate, and observed under transmission electron microscope (JEM-1010, JEOL, Japan).

### *Total Free Calcium in Circulation*

Serum calcium concentrations were determined by atomic absorbtion spectrophotometry (AA-6501, Shimadzu, Japan). Each group consisted of 20 rats.

### *Assay for Phosphorylase a*

Phosphorylase *a* was assayed in ovarian homogenates prepared in buffer containing 100 mmol/L NaF, 20 mmol/L EDTA, 0.5% (w/v) glycogen and 50 mmol/L glycylglycine. A portion (0.5 mL) of the homogenate was incubated with an equal volume of medium containing 100 mmol/L glucose 1-phosphate, 2% (w/v) glycogen, 0.3 mol/L NaF and 1 mmol/L caffeine (pH 6.1) at 37℃ for 30 min. Then, the reaction was stopped with 0.5 mL of 20% trichloroacetic acid followed by 3.5 mL of water[11]. The inorganic phosphate released from glucose 1-phophate was measured as previously described[12].

### *Assay for $Ca^{2+}$ and ATPase*

$Ca^{2+}$ and ATPase in ovarian homogenates were measured with a commercial kit provided by Nanjing Jiancheng Bioengineering Co. Ltd. (Nanjing, China).

### *ELISA for Calmodulin*

Ovarian homogenates prepared in buffer (20 mmol/L Tris-HCL,1 mmol/L EGTA, pH 7.8) were heated in boiling water bath at 95℃ for 4 min, then chilled on ice bath for 5 min and centrifuged at 10 000 × g for 30 min. The supernatants were collected for ELISA assay. In brief, 5 ug/mL recombinant human calmodulin (CaM, Nanjing General Hospital of Nanjing Command, Jiangsu,

China) as coating antigen was incubated at 4℃ for 72 h on 96-well microtiter plates treated with ultraviolet radiation to improve the coating effects. After the plates were washed, the blocking solution (10 g/L BSA) was added for another 72 h at 4℃. The rabbit CaM polyclonal antibody (Santa Cruz., CA) and specimens or standard substance were put in glass tubes for antigen-antibody reaction at 37℃ for 1h and then centrifuged at 10 000×g for 5 min. The supernatants were added into the coated plates at 37℃ for 1 h. The total concentration of calmodulin in specimens was detected using a peroxidase-conjugated goat antiserum to rabbit immunoglobulin (IgG), followed by addition of substrate. Results were obtained in optical density (OD) units on an ELISA plate reader (CERES908, Bio-TeK Instruments, Inc., Winooski, VT) at wavelength of 490 nm and converted to units by comparison with a standard curve from standard substance.

*Serum Progesterone*

The serum progesterone was measured using commercial RIA kits (Beijing North Institute of Biological Technology, China). The assay detection limit was 0.2 ng/mL. Inter- and intraassay coefficients of variation were <10% and <15%, respectively.

For standardization, the samples from animals were determined to be in diestrus at the time of blood collection according to vaginal smears.

*Protein Estimation*

The ovarian homogenates were centrifuged at 1000×g for 10 min. The supernatants were collected for protein quantitation by Bradford method with some modifications.

*Statistical Analysis*

Values in the tables and figures were expressed as $(\bar{x} \pm s)$. Comparison between two means was performed by Student's *t*-test. $P<0.05$ was considered statistically significant.

## RESULTS

*Effects of Fenvalerate on Ovary Ultrastructure*

As shown in Fig. 1b, expansion of endoplasmic reticulum in corpus luteum cells was observed in the 31.8 mg/kg group. Also, vacuolization and cristae loss in mitochondria were found in corpus luteum cells.

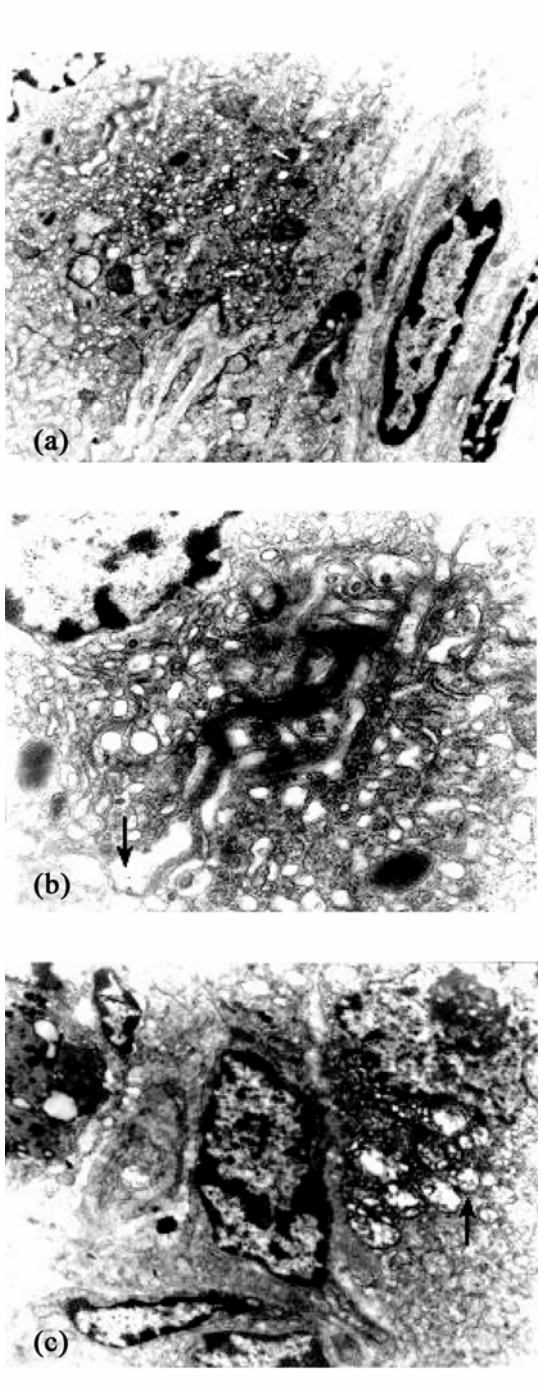


FIG. 1. Normal corpus luteum cells in control rats (a) (×6000), endoplasmic reticulum expansion (b) (arrowhead) (× 20 000), and racuolization and cristae loss (c) (arrowhead) (×6000), in rats treated with fenralerate.

*Effects of Fenvalerate on Serum Calcium Concentration*

The mean concentration of total free calcium ions in serum is shown in Table 1. Compared with the control, there was a significant increase in the 31.80 mg/kg group ($P<0.01$).

*Effects of Fenvalerate on Calcium Related Ovarian Enzyme*

The activities of phosphorylase *a* in ovary, representing an increased cytosolic free $Ca^{2+}$ after fenvalerate exposure in rats, showed a tendency to rise in the treated groups (Table 1). Fenvalerate increased the activities of phosphorylase *a* to 29%, 36%, and 44% of control respectively.

TABLE 1

Effects of Fenvalerate on $Ca^{2+}$, ATPase Phosphorylase *a*, Serum Calcium Concentration and Serum P4 Level ($\bar{x} \pm s$)

| Dose (mg/kg) | *n* | $Ca^{2+}$, ATPase (μmolPi. g. $prot^{-1}$. $hr^{-1}$) | *n* | Phosphorylase *a* (mgPi. g. $prot^{-1}$) | *n* | Serum Calcium (μg/mL) | *n* | P4 (ng/mL) |
|---|---|---|---|---|---|---|---|---|
| 0.00 | 7 | 103.9±10.6 | 6 | 45.3±2.4 | 20 | 221.8±17.3 | 9 | 2.2±1.8 |
| 1.91 | 7 | 80.6±5.1 | 7 | 58.5±14.3* | 20 | 225.8±22.3 | 9 | 1.9±1.3 |
| 9.55 | 5 | 99.7±17.7 | 5 | 61.5±13.3* | 20 | 231.0±29.0 | 7 | 1.5± 0.6 |
| 31.80 | 7 | 95.5±7.9 | 6 | 64.5±16.6* | 20 | 253.0±39.7* | 11 | 1.1±0.4* |

*Note.* *Statistically different from control using *t*-test, $P<0.05$.

$Ca^{2+}$, and ATPase, a transporter of calcium across biological membrane, were inhibited by fenvalerate. However, a marked dose-response relationship was not found (Table 1).

*Effects of Fenvalerate on Ovarian CaM*

The ovarian CaM was observed in rats after fenvalerate exposure by ELISA assay that reflected the total concentration of CaM including free and bound fractions. It was evaluated separately at each stage of the estrous cycle. The maximum concentration of CaM appeared in estrus both in the treated and untreated groups. A marked increase induced by fenvalerate was observed in estrus ($P<0.01$) (Fig. 2). The total concentration of CaM was approximately 2.5- and 3.1-fold higher than that of the control.

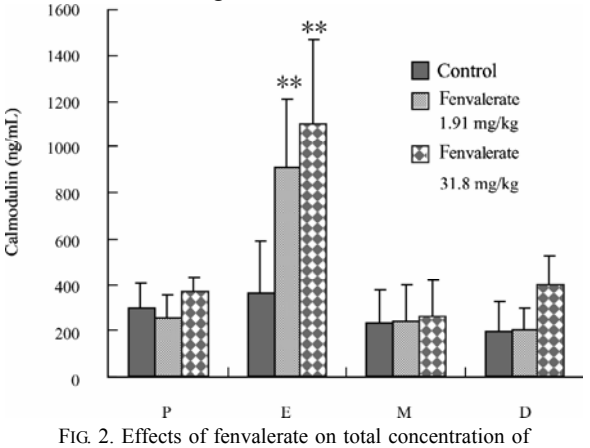


FIG. 2. Effects of fenvalerate on total concentration of calmodulin in ovaries of rats. Values are presented as $\bar{x} \pm s$ from five or seven animals in each estrous stage in each treatment. **Statistically different from corresponding control using *t*-test, $P<0.01$.

*Changes in Serum P4 Levels After Fenvalerate Exposure*

To evaluate the possible role of fenvalerate in endocrine disruption, we measured serum P4 concentrations in fenvalerate-exposed rats in diestrus. Fenvalerate induced a decrease in serum P4 concentrations, especially in 31.80 mg/kg group ($P<0.05$) (Table 1).

## DISCUSSION

Intracellular free calcium concentration is kept within the physiological limits by maintaining a delicate balance between influx and enflux of calcium. The cells can modulate $[Ca^{2+}]i$ both temporally and spatially. Such mechanisms include ligand- and voltage-gated ion channels, $Ca^{2+}$ pumps, and exchangers in both the plasma membrane and intracellular $Ca^{2+}$ storage-organelles[13].

Disruption of the mechanisms regulating calcium homeostasis is often an early event during the impairment of $Ca^{2+}$-mediated cell functions. The present results suggest that fenvalerate plays a role in the calcium homeostasis in ovary. The endoplasmic reticulum (ER) is the most dynamic store, it accumulates $Ca^{2+}$ by a pump, and releases it via channels gated by either inositol 1,4,5-trisphosphate ($IP_3$) or cyclic adenosine diphosphate ribose (cADPr). Mitochondrion is closely connected with the reticulum, and senses microdomains of high $Ca^{2+}$ close to IP3 or cADPr release channels[14]. In the present study, changes in both ER and mitochondrion in rat ovarian corpus luteum cells after a four-week fenvalerate exposure were observed. With the application of electron microscopy, we found expansion in ER and vacuolization and cristae loss in mitochondria.

Phosphorylase *a* is *a* valid indicator of fluctuations in cytosolic $Ca^{2+}$ level. Fenvalerate enhances the activity of ovarian phosphorylase *a*. The concentration of serum free calcium shows a rising tendency after fenvalerate exposure. The activity of

$Ca^{2+}$-ATPase in rat ovary, which is responsible for calcium transport, was also investigated in the study. However, we did not observe the dose-response relationship in the present study.

CaM has been shown to be the primary $Ca^{2+}$ mediator and serves as high affinity intracellular receptor[15]. The increased $Ca^{2+}$ can bind to CaM, and such binding induces a conformational change in CaM, exposing hydrophobic patches involved in interaction with and activation of target enzymes[16]. It is expressed in all eukaryotic cells, and rich in brain, testis and ovaries, *etc*. We found that the total concentration of CaM changed with estrous cycle. The total amount of CaM in ovaries of exposed rats increased in estrus period. Also, there was a difference between previous reports and our results. Rashatwar *et al.*[17] reported that cypermethrin and permethrin could inhibit CaM. However, Fakata *et al.*[18] suggested that the pyrethroids do not interfere with calmodulin-dependent signaling. These contradictions might be due to the differences in experimental methodology or species or organs.

It is reported that pyrethroids can largely bind to biological membranes with non-specific binding site[19]. Also, Sarkar *et al*.[20] reported that fenvalerate could interact with the membrane by localizing itself near the hydrophobic tail region of the lipid acyl chain and perturbs its ordered structure to make the membrane more fluid, suggesting that fenvalerate is likely to affect the activities of membrane proteins including various calcium channels. Certainly, the exact mechanism of fenvalerate underlying interruption of calcium homeostasis in ovarian cells needs further exploration.

Calcium can mediate various chemical-physical events, such as release of neurotransmitter, steroidogenesis, fertilization, and synthesis of DNA. Calcium and calcium-calmodulin system are involved in the gonadotropic regulation of granulosa cell steroidogenesis during follicular development. We evaluated the effects of fenvalerate on serum P4 concentrations in female rats in diestrus at four-week time points. Diestrus appears to be the most useful and best point for hormonal analysis for two reasons. Firstly, the number of animals is generally larger, due to its greater probability of occurrence. Secondly, the baseline is more stable facilitating detection of statistically significant changes[21]. A statistically significant decrease in serum P4 concentration was observed after exposure to 31.80 mg/kg fenvalerate. The results indicate that fenvalerate is capable of disrupting endocrine functions. It was reported that fenvalerate may be an estrogenic mimic compound[22-23]. Studies about the effects of 17β-estradiol on serum hormone concentration in female rats indicate that serum P4 concentration decreases at each stage of estrous cycle[21]. Therefore, the effects of fenvalerate might be explained as an estrogen mimic.

In conclusion, fenvalerate can interrupt calcium homeostasis in ovary, and is able to disrupt endocrine function by inhibiting serum P4 concentration. There might be a relationship between calcium or $Ca^{2+}$/CaM system and changes in steroid level induced by fenvalerate. Further and detailed studies are necessary to elucidate this hypothesis. The mechanisms of fenvalerate underlying interruption of calcium homeostasis in ovary and its downstream events are deserved to be studied in the future.

## REFERENCES


1. Wilson R, Spengler J (1996). Particles in our air: concentrations and health effects. Harvard University Press, Boston.
2. Kan H, Chen B, Chen C, *et al*. (2004). An evaluation of public health impact of ambient air pollution under various energy scenarios in Shanghai, China. *Atmospheric Environment* **38**(1), 95-102.
3. Kan H, Chen B (2002). Meta analysis of exposure-response functions of air particulate matter and adverse health outcomes in China. *Journal of Environment and Health* **19**(6), 422-424.
4. Teng E, Hu W, Wu G (1999). The composing characteristics of elements in coarse and fine particle in air of the four cities in China. *China Environmental Science* **19**, 238-242.
5. Dockery D W, Pope C A, Xu X, *et al*. (1993). An association between air pollution and mortality in six U.S. Cities. *N Eng J Med* **329**, 1753-1759.
6. Pope C A, Thun M J, Namboodiri M M, *et al*. (1995). Particulate air pollution as a predictor of mortality in a prospective study of U.S. adults. *Am J Resp Crit Care Med* **151**, 669-674.
7. Pope C A, Burnett R T, Thun M J, *et al*. (2002). Lung cancer, cardiopulmonary mortality, and long-term exposure to fine particulate air pollution. *JAMA* **287**(9), 1132-1141.
8. Jin L B, Qin Y, Xu Z (2000). Relationship between air pollution and acute and chronic respiratory disease in Benxi. *Journal of Environment and Health* **17**, 268-270.
9. Ma H B, Hong C J (1992). Effects of particulate air pollution on respiratory disease. *Chinese Journal of Public Health* **11**, 229-232.
10. Spix C, Anderson H R, Schwartz J, *et al*. (1998). Short-term effects of air pollution on hospital admissions of respiratory diseases in Europe: a quantitative summary of APHEA study results. Air Pollution and Health: a European Approach. *Arch Environ Health* **53**(1), 54-64.
11. Wordley J, Walters S, Ayres J G (1997). Short term variations in hospital admissions and mortality and particulate air pollution. *Occup Environ Med* **54**(2), 108-16.
12. Prescott G J, Cohen G R, Elton R A, *et al*. (1998). Urban air pollution and cardiopulmonary ill health: a 14.5 year time series study. *Occup Environ Med* **55**(10), 697-704.
13. Thurston G D, Ito K, Hayes C G, *et al*. (1994). Respiratory hospital admissions and summertime haze air pollution in Toronto, Ontario: consideration of the role of acid aerosols. *Environ Res* **65**(2), 271-290.
14. Schwartz J (1994a). Air pollution and hospital admissions for the elderly in Detroit, Michigan. *Am J Respir Crit Care Med* **150**(3), 648-655.
15. Schwartz J (1994b). Air pollution and hospital admissions for the elderly in Birmingham, Alabama. *Am J Epidemiol* **139**(6), 589-598.
16. Schwartz J (1994c). $PM_{10}$, ozone, and hospital admissions for the elderly in Minneapolis-St. Paul, Minnesota. *Arch Environ*

*Health* **49**(5), 366-374.
17. Schwartz J (1995). Short term fluctuations in air pollution and hospital admissions of the elderly for respiratory disease. *Thorax* **50**(5), 531-538.
18. Schwartz J (1996). Air pollution and hospital admissions for respiratory disease. *Epidemiology* **7**(1), 20-28.
19. Schwartz J, Spix C, Touloumi G, *et al*. (1996). Methodological issues in studies of air pollution and daily counts of deaths or hospital admissions. *J Epidemiol Community Health* **50**(Suppl 1), 3-11.
20. Burnett R T, Cakmak S, Brook J R, *et al*. (1997). The role of particulate size and chemistry in the association between summertime ambient air pollution and hospitalization for cardiorespiratory diseases. *Environ Health Perspect* **105**(6), 614-620.
21. Medina S, Tertre A, Dusseux E (1997). Evaluation des Risques de la Pollution Urbaine sur la Santé (ERPURS). Analyse des liens à court terme entre pollution atmoshérique et santé. Resultats 1991-1995, Conseil Regional d'Ile de France.
22. Poloniecki J D, Atkinson R W, Leon A P, *et al*. (1997). Daily time series for cardiovascular hospital admissions and previous day's air pollution in London, UK. *Occup Environ Med* **54**(8), 535-540.
23. Schwartz J, Morris R (1995). Air pollution and hospital admissions for cardiovascular disease in Detroit, Michigan. *Am J Epidemiol* **142**(1), 23-35.
24. Schwartz J (1997). Air pollution and hospital admissions for cardiovascular disease in Tucson. *Epidemiology* **8**(4), 371-377.
25. Xu X, Dockery D W, Christiani D C, *et al*. (1995). Association of air pollution with hospital outpatient visits in Beijing. *Arch Environ Health* **50**(3), 214-220.
26. Wei F, Hu W, Teng E (2000). Relation analysis of air pollution and children's respiratory system disease prevalence. *China Environmental Science* **20**(3), 220-224.
27. Dusseldorp A, Kruize H, Brunekreef B, *et al*. (1995). Association of $PM_{10}$ and airborne iron withrespiratory health of adults living near a steel factory. *Am J Respir Crit Care Med* **152**, 1032-1039.
28. Hiltermann T J, Stolk J, Zee S C, *et al*. (1998). Asthma severity and susceptibility to air pollution. *Eur Respir J* **11**, 686-693.
29. Neukirch F, Segala C, Moullec Y, *et al*. (1998). Short-term effects of low-level winter pollution on respiratory health of asthmatic adults. *Arch Environ Health* **53**, 320-328.
30. Ostro B D, Lipsett M J, Wiener M B, *et al*. (1991). Asthmatic responses to airborne acid aerosols. *Am J Public Health* **81**(6), 694-702.
31. Pope C A, Dockery D W, Spengler J D, *et al*. (1991). Respiratory health and $PM_{10}$ pollution: A daily time series analysis. *Am Rev Respir Dis* **144**, 668-674.
32. Kunzli N, Medina S, Kaiser R, *et al*. (2001). Assessment of deaths attributable to air pollution, should we use risk estimates based on time series or on cohort studies? *Am J Epidemiol* **153**(11), 1050-1055.
33. Stieb D M, Judek S, Burnett R T (2002). Meta- analysis of time- series studies of air pollution and mortality: effects of gases and particles and the influence of cause of death, age, and season. *J Air Waste Manag Assoc* **52**, 470-484.

(*Received May* 28, 2004 *Accepted September* 29, 2005)