

Protective Effects of Eicosapentaenoic Acid on Genotoxicity and Oxidative Stress of Cyclophosphamide in Mice

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Received 22 May 2009; revised 7 September 2009; accepted 17 September 2009

ABSTRACT: The aim of this article is to elucidate the mechanism by which eicosapentaenoic acid (EPA) acts against cyclophosphamide (CP)-induced effects. The prevalence of micronuclei, the extent of lipid peroxidation, and the status of the antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) in both liver and serum of mice were used as intermediate biomarkers of chemoprotection. Lipid peroxidation and associated compromised antioxidant defenses (CAT and GPX) in CP treated mice were observed in the liver, serum, and were accompanied by increased prevalence of micronuclei in bone marrow. The number of MN was significantly different ($p < 0.01$) between the groups treated with CP (group III, IV, V, VI) and the solvent control (group II) ($3.2 \pm 0.7\%$). There was a dose-dependent reduction in formation CP induced micronuclei by treatment with 100, 200, or 300 mg EPA/kg BW mice. Activities of SOD, CAT, and extent of lipid peroxidation were statistically different in liver cells of mice exposed to EPA only with CP compared with the CP group (group III). The present findings imply that EPA may be a potential antigenotoxic, antioxidant and chemopreventive agent and could be used as an adjuvant in chemotherapeutic applications. © 2010 Wiley Periodicals, Inc. *Environ Toxicol* 00: 000–000, 2010.

Keywords: antioxidant enzymes; lipid peroxidation; micronucleus (MN); polyunsaturated fatty acids (PUFAs)

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Contract grant sponsor: National Basic Research Program of China (973 program).

Contract grant number: 2008CB418102.

Contract grant sponsor: Jiangsu Social Development Project Foundation

Contract grant number: BS2007049.

Contract grant sponsor: Discovery Grant from the National Science and Engineering Research Council of Canada.

Contract grant number: Project # 326415-07.

Contract grant sponsor: Western Economic Diversification Canada.

Contract grant number: Project # 6578 and 6807.

Contract grant sponsor: Canada Foundation for Infrastructure.

Contract grant sponsor: Canada Research Chair program and an at large Chair Professorship at the Department of Biology and Chemistry and Research Centre for Coastal Pollution and Conservation, City University of Hong Kong (to J.P.G.).

Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/tox.20546

INTRODUCTION

Polyunsaturated fatty acids (PUFAs) are important as an energy source and as membrane components, and have been shown to have biological effects at the tissue, cellular, and molecular levels (Ross et al., 1999), and control regulation of some genes. PUFAs are of interest since they can have a range of pharmaceutical applications (Simopoulos, 2002). The 5, 8, 11, 14, 17-eicosapentaenoic acid (EPA), a 20-carbon polyunsaturated fatty acid with five double bonds, is one of the major dietary PUFAs. High-purity EPA was studied rather than a cocktail of fatty acids such as fish oil.

During treatment with some anticancer drugs, genotoxicity of these drugs can result in unwanted side effects, such as secondary tumors in nontumor cells. For example, cyclophosphamide (CP), an alkylating drug, has been in clinical use for the treatment of malignant and nonmalignant disorders for over 40 years. However, toxic metabolites of CP can cause toxicity toward normal cells (Fraiser et al., 1991). The toxicity of CP is primarily due to the damage to DNA (Brookes, 1990). Therefore it is important to cotreat patients with an agent that can avoid side effects of CP in cancer treatment.

The micronucleus (MN) test has been used for assessing genotoxicity (Hayashi et al., 2000). Classically, a MN is defined as a small extranuclear chromatin body originating from an acentric fragment or whole chromosome lost from the metaphase plate. Therefore, MN frequencies have been considered reliable biomarkers of both chromosome breakage and chromosome loss (Le et al., 2003; Norppa and Falck, 2003).

Some chemopreventive agents exert their protective effects against the deleterious effects of genotoxic carcinogens by scavenging reactive oxygen species (ROS) and enhancing host antioxidant defense systems (Weisburger, 2001). Tolerance and protective mechanisms have evolved to scavenge free radicals and peroxides generated during various metabolic reactions. In this study we investigated the ability of EPA to modulate the genetic damage induced by CP in the mouse erythropoietic system by determining the frequency of micronuclei in immature erythrocytes of bone marrow. In order to elucidate the mechanism by which EPA acts against CP-induced effects, the extent of lipid peroxidation and activities of certain key antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX) were also investigated in mouse liver and serum.

METHODS

Chemicals

The 5, 8, 11, 14, 17-eicosapentaenoic acid (EPA, purity higher than 90%) was extracted from the marine microalgae

Pavlova viridis cultured in Jiangning district, Nanjing, China. The purity of the fatty acid was analyzed using a HP6890 gas chromatograph (Hewlett-Packard, Avondale, PA) equipped with a flame-ionization detector and a silica capillary column (30 m × 0.25 mm × 0.2 μm, INNOWAX, Hewlett-Packard). The flow of carrier gas was helium and a 1-μL methyl ester solution of sample was injected for each analysis. The temperature program was as follows: the initial temperature was 180°C, and rose to 200°C at a rate of 2°C/min, then from 200°C to 250°C at a rate of 5°C/min. The final oven temperature of 250°C was kept for 5 min. Fatty acids were identified from retention times of known standards (Sigma) and mass spectra acquired by gas chromatography-mass spectrometry. CP was obtained from Jiangsu Hengrui Pharmaceutical Co. Ltd (Nanjing). Poloxamer 188 was purchased from Nanjing Well Chemical Co. Ltd. Poloxamer 188 was used in this study because of its application in drug delivery (Prancan et al., 1980; Yun et al., 1999). If EPA is a feasible option in clinical treatment, P188 could be used to enhance the bioavailability of EPA.

Animals and Treatment

Seventy healthy Kunming mice (*Mus musculus*) weighting 20 ± 2 g, about 3 weeks old were purchased from the Experimental Animal Center of Qinglong Mountain, Jiangning district, Nanjing. Animals were housed in polycarbonate boxes (five mice/box) with steel wire tops. They were maintained in a controlled atmosphere with a 12:12 h dark: light cycle, a temperature of 22 ± 2°C and a humidity of 50–70% with free access to standard mouse pellets and freshwater during the experimental period. Mice were randomly divided into seven groups of ten animals (five males, five females) each and allowed to acclimatize for 5 days before treatment. The EPA concentrations of 100, 200, and 300 mg/kg body weight respectively for treatment were selected on a preliminary experiment and by taking into consideration of a published report (Cao et al., 2005). Body weights of all the animals were recorded weekly. The mice were treated according to NIH animal care guidelines, and then killed in accordance with the internationally accepted principles and approved by the Ethical Committee of the University of Nanjing.

- Group I: normal control; received distilled water (10 mL/kg, BW) by gavage for 21 days.
- Group II: solvent control; received solvent Poloxamer 188 (P188, 10 mL/kg, BW) by gavage for 21 days.
- Group III: positive control, treated with a single dose of cyclophosphamide (CP, 40 mg/kg, BW, i.p.) in saline, 1 h after the last dose of solvent P188 (10 mL/kg, BW for 21 days by gavage).

- Group IV: treated with a single i.p. dose of CP, 1 h after the last dose of EPA (100 mg/kg, BW for 21 days) in P188 (10 mL/kg, BW) by gavage.
- Group V: treated with a single i.p. dose of CP, 1 h after the last dose of EPA (200 mg/kg, BW for 21 days) in P188 (10 mL/kg, BW) by gavage.
- Group VI: treated with a single i.p. dose of CP, 1 h after the last dose of EPA (300 mg/kg, BW for 21 days) in P188 (10 mL/kg, BW) by gavage.
- Group VII: treated by gavage with EPA (300 mg/kg, BW for 21 days) in P188 (10 mL/kg, BW).

Determination of Micronuclei in Bone Marrow

After mice were treated with CP for 30 h, blood samples were taken from the mice orbital cavity and then mice were killed by cervical dislocation. Bone marrow cells of both femurs were flushed through a 21-gauge needle using a disposable syringe into a centrifuge tube containing 1 mL of fetal calf serum (FCS). This suspension was centrifuged at 1000 rpm for 5 min. The pellet of cells was resuspended in a drop of fresh FCS and smears were prepared on clean glass slides and air-dried. Four slides were prepared for each mouse. The dried, evenly spread bone marrow smears were fixed with methanol for 5 min and stained with Giemsa at pH 6.8 (Muller and Streffer, 1994; Narayana et al., 2002). A total of 1000 polychromatic erythrocytes (PCE) were screened per animal by the same observer for determining the frequency of polychromatic erythrocytes containing micronuclei (MnPCEs) and the PCE/(NCE + PCE) ratio (in which NCE is normochromatic erythrocytes). The criteria for scoring micronuclei were given in detail elsewhere (Cllet, 1993).

Lipid Peroxidation and Antioxidant Enzymes

The effects of treatments on lipid peroxidation and antioxidant enzymes were determined by corresponding assays in every group. Blood was separated by centrifugation at 1000 rpm for 10 min. Livers were removed from mice and perfused immediately with ice-cold saline (0.9% sodium chloride) and homogenized in chilled phosphate buffer (0.1 M, pH 7.4) using JY92-II homogenizer (Ningbo, China). The homogenate was centrifuged at 4000 rpm for 5 min at 4°C (Beckman J2-MC). Products of lipid peroxidation were estimated by measuring the concentration of malondialdehyde (MDA) expressed as thiobarbituric acid reactive substances (TBARS) according to the methods of Heath and Parker (Heath and Parker, 1968). Total superoxide dismutase activity (SOD, EC 1.15.1.1) was determined by the ferric cytochrome *c* method using xanthine and xanthine oxidase to generate superoxide radicals, and a unit of activ-

ity was defined by McCord and Fridovich (McCord and Fridovich, 1969). Catalase activity (CAT, EC.1.11.1.6) was measured spectrophotometrically with a Hitachi U-3000 by measuring the decrease of absorbance at 240 nm due to H₂O₂ decomposition (Aebi, 1984). Glutathione peroxidase (GPX, EC.1.11.1.9) activity was measured in the presence of added glutathione (Flohe and Gunzler, 1984). Protein content of homogenates was determined by reaction with Coomassie Blue dye, using bovine serum albumin as the standard, in a VIS-7220 spectrophotometer (Bradford, 1976). The activities of the various enzymes in mouse liver were expressed as U/mg of protein.

Statistical Analysis

The data were expressed as mean \pm S.D. Statistical significances of differences among treatments were determined by use of one-way analysis of variance and covariance (ANOVA), followed by Tukey's pair-wise comparisons at significance level of 0.05.

RESULTS

Micronuclei in Bone Marrow

There was a significantly greater frequency of MnPCEs in CP-treated mice (group III) compared with controls (group I and II). The frequency of MN and the ratio of PCE to NCE + PCE in bone marrow cells of different groups are summarized in Table I. Administration of 300 mg EPA/kg, BW resulted in a MN frequency of 11.7‰ which was significantly ($p < 0.05$) less than the 23.2‰ observed in the solvent + CP (group III) treatment. The number of MN was significantly different between the groups treated

TABLE I. Effects of EPA on the formation of cyclophosphamide (CP) induced micronucleated polychromatic erythrocytes (MnPCEs) and ratio of PCE/(NCE + PCE) in mouse bone marrow

Group	Treatment	MnPCEs (‰) ^a	PCE/(NCE + PCE)
I	Control, distilled water	2.7 \pm 0.8	0.5 \pm 0.1
II	Solvent, P188	3.2 \pm 0.7	0.4 \pm 0.1
III	P188 + CP	23.2 \pm 1.8 ^b	0.5 \pm 0.1
IV	100 mg EPA + CP	26.3 \pm 2.2 ^b	0.5 \pm 0.1
V	200 mg EPA + CP	18.4 \pm 1.4 ^b	0.5 \pm 0.1
VI	300 mg EPA + CP	11.7 \pm 1.2 ^{b,c}	0.4 \pm 0.2
VII	300 mg EPA	3.4 \pm 1.1	0.5 \pm 0.1

PCE, polychromatic erythrocytes; NCE, normochromatic erythrocytes; MnPCEs, micronucleated polychromatic erythrocytes.

^a Values represent mean \pm S.D. for each group of 10 mice.

^b Significantly different from group II ($p < 0.01$).

^c Significantly different from group III ($p < 0.05$).

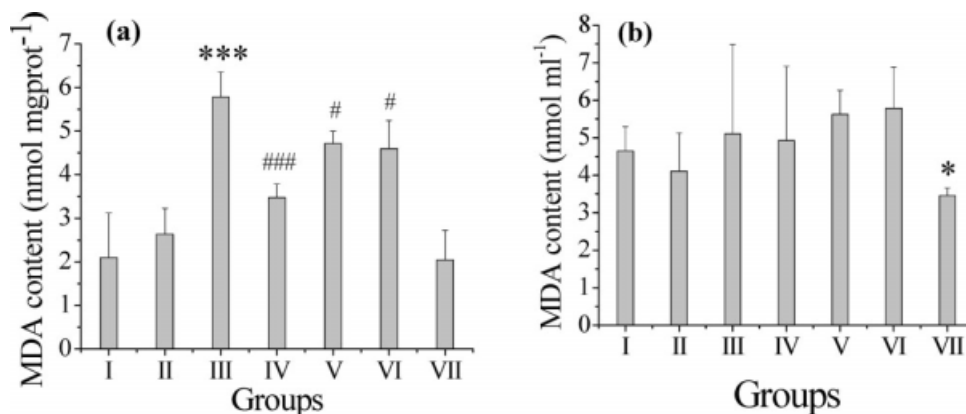


Fig. 1. Concentrations of the lipid peroxidation product (MDA) measured as thiobarbituric acid reactive substances (TBRS) in mouse liver (a) and serum (b) exposed to different doses of EPA. Results are expressed as the mean \pm SD ($n = 10$). *** Significantly different from group II ($p < 0.001$), # Significantly different from group III ($p < 0.05$), ### Significantly different from group III ($p < 0.001$).

with CP (group III, IV, V, VI) and the solvent control (group II) ($p < 0.01$). The number of spontaneous MN observed in unexposed, control normal mice was $2.7 \pm 0.8\%$.

Evaluation of erythropoietic cytotoxicity is a key component of safety assessment in new drug development and PCE counts in peripheral blood are the most popular and convenient method of monitoring erythropoiesis. The occurrence of fewer immature erythrocytes (PCE) relative mature or normochromatic erythrocytes (NCE) is considered to be an indicator of mutagen-induced cytotoxicity (Suzukiet al., 1989). Therefore, the ratio of PCE to NCE is one index of cytotoxicity that is routinely included in micronucleus tests to assess the mutagenicity of chemicals to mammals (Criswell et al., 1998; Celik et al., 2003). The

values for this index were similar in groups treated with distilled water (group I), P188 solvent (group II) or 300 mg EPA/kg, BW (group VII). There was no statistically significant difference among the PCE/(NCE+PCE) ratios relative to the vehicle control (group I) and solvent (group II) of any of the EPA treatments. This result indicates a lack of toxicity to bone marrow.

Lipid Peroxidation

Group I and group VII didn't show significant changes in MDA content while group III spiked 119.4% higher compared with group II. Concentrations of MDA in groups IV, V, and VI, were significantly less (32.4%, 18.4%, and 20.5%, respectively) than those in group III, (Fig. 1).

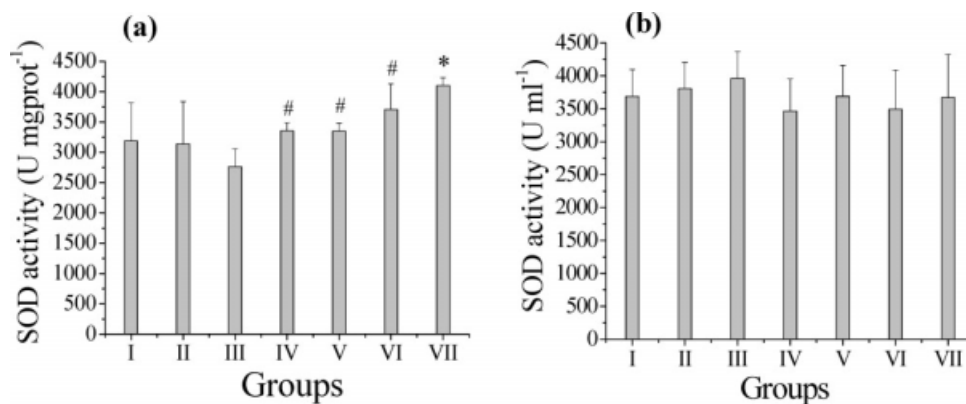


Fig. 2. SOD activity in mouse liver (a) and serum (b). Results are expressed as the mean \pm SD ($n = 10$). * Significantly different from group II ($p < 0.05$), # Significantly different from group III ($p < 0.05$).

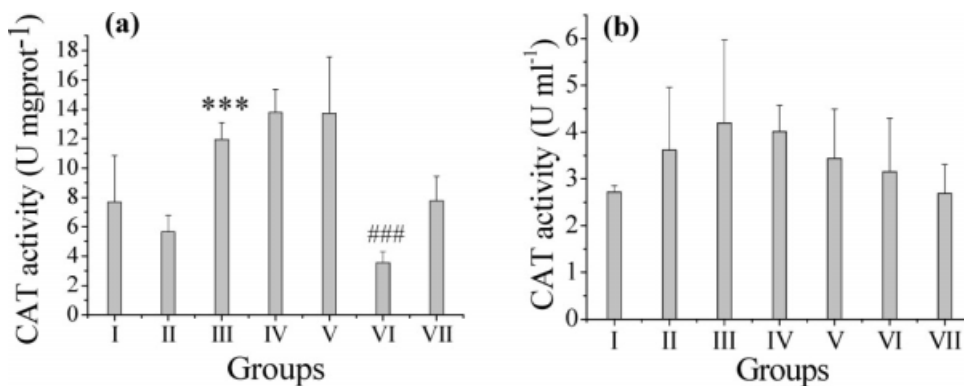


Fig. 3. CAT activity in mouse liver (a) and serum (b). Results are expressed as the mean \pm SD ($n = 10$). *** Significantly different from group II ($p < 0.001$), ### Significantly different from group III ($p < 0.001$).

Groups I, III, and VII did not show significant differences in MDA content compared with group II in sera. And the CP-treated groups (III, IV, V, and VI) had similar MDA contents in serum.

Antioxidant Enzyme Activities

In livers, the antioxidant enzyme activities varied. For SOD activities, group VII showed a significant increase compared with group II; significant increases in SOD activities were detected in groups IV, V and VI compared with group III (21.4%, 21.2% and 34.0%, respectively, see Fig. 2). CAT activities remained similar among groups I, II and VII but group III experienced a very significant increase (111.0%, $p < 0.001$) compared with group II; a very significant decrease (70.3%, $p < 0.001$) was found in group VI when compared with group III (Fig. 3). GPX activities were similar between groups II and VII but significantly lower in group I (19.4%, $p < 0.001$) and higher in group III (11.0%, $p < 0.05$), no significant difference was found among groups III, IV, V, and VI (Fig. 4). Compared with

the antioxidant enzyme activities in livers, no significant changes were observed in serum.

DISCUSSION

Based on the results of the micronucleus assay (Muller and Streffer, 1994), chromosomal damage was the major cause for the appearance of micronuclei due to exposure to CP and as an indicator of genotoxic insult to nuclei (Salam et al., 1993) and treatment with EPA resulted in significantly less toxicity. Exposure to 100 mg/kg CP, BW caused a statistically significant greater rate of breaks in single strand DNA in bone marrow cells and provided further evidence by using the comet assay to assess the genotoxicity of CP (Zhang et al., 2008). The fact that administration of 40 mg/kg BW (group III) resulted in a significantly greater frequency of MnPCEs in bone marrow cells than did distilled water or solvent groups (I and II) (Table I) demonstrates the genotoxicity of CP. Administration of EPA for 21 days inhibited formation of micronuclei in mouse bone

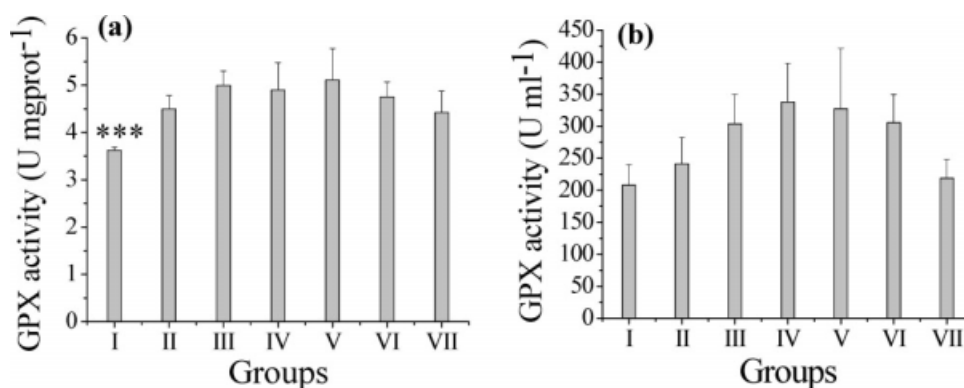


Fig. 4. GPX activity in mouse liver (a) and serum (b). Results are expressed as the mean \pm SD ($n = 10$). *** Significantly different from group II ($p < 0.001$).

marrow cells by CP. EPA significantly reduced the frequency of MnPCEs and exhibited protection and anticlastogenic effects against the effects of CP. Exposure to the greatest concentration of EPA could have triggered apoptosis mediated by increased hydroxyl radical production accompanied by exhaustion of the liver catalase. Similar decreases in the MnPCEs induced by CP have been described for vitamin C (Rompelberg et al., 1995) and stem, leaf of *Panax ginseng* (Zhang et al., 2008).

The results presented here suggest that CP-induced oxidative stress is a contributing factor for the greater frequency of bone marrow micronuclei observed in the present study. Several studies have demonstrated a positive correlation between degree of lipid peroxidation and genotoxicity as reflected by increased micronuclei formation (Rompelberg et al., 1995; Mayer et al., 2000; Kumaraguruparan et al., 2005). The effects of CP on the mouse livers observed under our experimental conditions could be associated with the increase in lipid peroxidation, as measured by MDA production. Other studies have indicated that CP has a pro-oxidant character (Dumontet et al., 2001; Bhattacharya et al., 2003). Administration of CP leads to generation of oxidative stress in liver, lungs, and serum of mice and rats with a resulting decrease in the activities of antioxidant enzymes and increase in lipid peroxidation in these tissues. The greater concentration of MDA caused by exposure to CP indicates greater lipid peroxidation. The fact that groups treated with EPA resulted in lesser concentrations of MDA suggests that EPA resulted in fewer MN due to a lessening of peroxidation in livers of mice. The greater lipid peroxidation, which is considered to be an indicator of increased oxidative damage, suggests that this was a primary effect caused by CP in mouse liver. Some anticancer drugs including CP are known to exert their cytotoxic effects by a free radical mediated mechanism (Subapriya et al., 2004).

Oxidative stress arising from overproduction of ROS and the breakdown of antioxidant defenses is documented to induce chromosomal breakage and formation of bone marrow micronuclei (Simic, 1994). It has been suggested that clastogenic factors are released by cells exposed to oxidative stress (Emerit et al., 1995). Free radical scavengers, including naturally occurring compounds such as vitamin C, have been found to reduce or neutralize the activity of such reactive oxygen species (Subapriya et al., 2004). Our results showed that administration relatively great doses of EPA resulted in greater SOD activity in the liver, and that these elevated SOD activities can help to protect against the genotoxicity of CP in bone marrow cells. These results support the hypothesis that CP-induced genotoxicity and cytotoxicity to bone marrow cells may be partially repaired by antioxidant activity. The protective activity of the polyunsaturated fatty acid EPA may be due to its inhibitory action on several enzymes or its blocking effect on oxidative damage.

CONCLUSION

In summary, the results of this study demonstrate that EPA protects against CP-induced genotoxicity in mice. Our findings are important because EPA can be used as a natural dietary supplement to counteract the cytotoxic effects resulting from exposure to mutagens and carcinogens through drugs, diet, and environment. The chemopreventive actions of EPA in this effect may be partially attributed to its elevation of the levels of enzymatic antioxidants (SOD and CAT). The present findings imply that EPA may be a potential antigenotoxic, antioxidant and chemopreventive agent and could be used as an adjuvant in chemotherapeutic applications.

The authors thank Mike Newton, D.S. Li, Dr. D.H. Cao, and H.T. Ge for their kind advice.

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