Chemopreventive effects of early-stage and late-stage supplementation of vitamin E and selenium on esophageal carcinogenesis in rats maintained on a low vitamin E/selenium diet

Hui Yang, Jin Fang, Xudong Jia, Chi Han, Xiaoxin Chen, Chung S. Yang* and Ning Li*

Introduction

Esophageal cancer is one of the most common malignancies. Esophageal squamous cell carcinoma (ESCC) remains the major histological type worldwide, though adenocarcinoma has surpassed ESCC as the most prevalent type in the Western countries (1,2). 

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Abbreviations: AA, arachidonic acid; COX2, cyclooxygenase 2; ESCC, esophageal squamous cell carcinoma; 5LOX, 5-lipoxygenase; LTB4, leukotriene B4; NMBzA, N-nitrosomethylbenzylamine; 8OH-dG, 8-hydroxy-2’-deoxyguanosine; PGE2, prostaglandin E2; Se, selenium; Ve, vitamin E.

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Demonstrated that supplementation with Ve, Se and β-carotene significantly decreased the risk of esophageal and gastric cardia cancer (10–12). A follow-up study indicated that the beneficial effects of the supplementation on mortality were still evident 10 years after the cessation of supplementation (13). In addition, a preventive effect of supplementation with Ve, Se and β-carotene against esophageal cancer was observed in subjects who entered in the trial at ages <55 years old but not in older subjects (13). Furthermore, a nested case-control study on this population demonstrated that esophageal cancer risk was related to serum levels of Ve and Se, rather than that of β-carotene (14,15). The baseline levels of plasma α-tocopherol for the lower two quartiles were ≤8.0 μg/ml and ≤70 ng/ml. On the other hand, a parallel trial with antioxidants and other nutrients, started in 1985 in Linxian, failed to demonstrate a protective effect against esophageal and gastric cardia cancer among adults with esophageal dysplasia (16). Taken together, these results suggest that supplementation with antioxidant nutrients was effective among younger subjects who were less probably to have severe dysplastic lesions but ineffective in older subjects who were more probably to have more severe lesions. Consistent with this concept is the result of a subsequent intervention study with selenomethionine, which showed beneficial effects in patients with mild esophageal dysplasia but not in those with severe esophageal dysplasia (17).

It is unclear whether the ‘ timing ’ of the intervention period can account for the inconsistent results from other chemoprevention trials with nutrients. In the Alpha-Tocopherol Beta-Carotene Cancer Prevention Study, Ve and β-carotene failed to prevent upper aerodigestive tract cancers (18). The Se and Ve Cancer Prevention Trial (SELECT) demonstrated that Se, Ve or their combination at the tested doses and formulations did not prevent prostate cancer in a generally healthy and heterogenous population of men who were well nourished in Ve and Se (with median plasma levels of 12 μg α-tocopherol and 135 ng Se/ml) (19,20). Results from some clinical trials even raise concern that certain micronutrients could promote growth of pre-existing tumors or precancerous lesions (21,22). Possible promotion of colorectal cancer by folic acid supplementation is an example (23). An animal study suggested that folate supplementation at the early stage (prior to the existence of preneoplastic lesions) could inhibit colorectal cancer formation, but supplementation at the late stage could promote carcinogenesis (23,24). Ulrich et al. (25) proposed that the efficacy of cancer chemoprevention by nutrients may be time selective during the multistages of carcinogenesis. This concept is consistent with the above mentioned results that Ve/Se supplementation decreased esophageal cancer deaths among participants <55 but may have no effect or produce an opposite effect among those aged ≥55 (13). Therefore, it is important to understand how the timing of Ve/Se supplementation or deficiency affects esophageal carcinogenesis as well as the underlying molecular mechanism.

β-Nitrosomethylbenzylamine (NMBzA) is a potent esophageal carcinogen in rodents (26). Because of its organ specificity and possible role as a human carcinogen, NMBzA has been used extensively to study the development and progression of esophageal cancer in rodents. NMBzA-induced papilloma and carcinoma formation in the Fischer 344 rat has proven to be a valuable animal model for chemopreventive studies (27,28). Using this model, we tested the preventive effect of Ve/Se supplementation on esophageal carcinogenesis in rats maintained on low Ve/Se diet. We were particularly interested in the chemopreventive effects of early versus late stage Ve/Se supplementation on the development of esophageal cancer.

High plasma 5α-tocopherol in this population reported in 1984 was 700 μg/dl (5), which is much lower than the normal levels of 55 μg/dl in Shanghai (9). The General Population Nutrition Intervention Trial conducted from 1985 to 1991 in this area demonstrated that supplementation with Ve, Se and β-carotene significantly decreased the risk of esophageal and gastric cardia cancer (10–12). A follow-up study indicated that the beneficial effects of the supplementation on mortality were still evident 10 years after the cessation of supplementation (13). In addition, a preventive effect of supplementation with Ve, Se and β-carotene against esophageal cancer was observed in subjects who entered in the trial at ages <55 years old but not in older subjects (13). Furthermore, a nested case-control study on this population demonstrated that esophageal cancer risk was related to serum levels of Ve and Se, rather than that of β-carotene (14,15). The baseline levels of plasma α-tocopherol for the lower two quartiles were ≤8.0 μg/ml and ≤70 ng/ml. On the other hand, a parallel trial with antioxidants and other nutrients, started in 1985 in Linxian, failed to demonstrate a protective effect against esophageal and gastric cardia cancer among adults with esophageal dysplasia (16). Taken together, these results suggest that supplementation with antioxidant nutrients was effective among younger subjects who were less probably to have severe dysplastic lesions but ineffective in older subjects who were more probably to have more severe lesions. Consistent with this concept is the result of a subsequent intervention study with selenomethionine, which showed beneficial effects in patients with mild esophageal dysplasia but not in those with severe esophageal dysplasia (17).

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Materials and methods

Animals, diets and treatment

Two hundred and fifty-nine-week-old male F344 rats were purchased from Vitalriver (Beijing, China) and randomized into five experimental groups (Groups A–E). Rats were housed in a controlled environment with a 12 h light/dark cycle and given water ad libitum. Two different diets were prepared by Keaoxili Diet Co. (Beijing, China), normal diet and low Ve/Se diet. The normal diet was produced according to AIN-93M formula, which contained 80 IU/kg π-tocopherol and 0.15 mg/kg Se (29). The low Ve/Se diet was made with the same formula but contained 46 IU π-tocopherol and 0.05 mg Se/kg diet. The Ve level was lower than those in the AIN93N diet, but still higher than the estimated requirement of 18 mg π-tocopherol, but the Se level was lower than the requirement of 0.15 mg Se per kg diet for the growth rats (30). The purpose was to mimic the low Ve/Se nutritional status in the human populations such as that in Linxian, China, where low nutrient intakes were found for Ve (78% recommended dietary allowance) and Se (66% recommended dietary allowance) (8). Such low nutritional status was believed not to impact the basic physiological functions of animals and humans.

After acclimatization for 2 weeks, rats in Groups A, B, C and D were treated with NMBzA (Ash Stevens, Detroit, MI), solubilized in 20% dimethyl sulfoxide per water, at 0.35 mg/kg body weight, subcutaneously, three times per week for 5 weeks (Figure 1A). Group E was given the vehicle as negative control. Ten rats from each of Groups A and D were killed at Weeks 10, 13 and 15 to assess the development of esophageal lesions. Rats were maintained on the low Ve/Se diet as the control group and other groups were put on the normal AIN-93M diet at the early stage (Week 0–10), late stage (Week 11–25) or throughout the entire experiment (Week 0–25). Body weight and food intake were measured once a week during the experiment.

Nutritional analysis

At Weeks 0, 5, 15 and 25, 0.5 ml blood samples of 10 rats in each group were collected from the retro-orbital vein after anesthesia with ether. Plasma was stored at −80°C until analysis. π-Tocopherol levels were determined by high-performance liquid chromatography. In brief, lipids were extracted with hexane from plasma and separated in a reverse phase C18 Atlantis column in an Alliance 2695 high-performance liquid chromatography system (Waters, Milford, MA). The column was equilibrated in mobile phase composed of 50 mM sodium perchlorate in a mixture of methanol: water (96:4) and eluted at a flow rate of 1.2 ml/min. π-Tocopherol was detected with a ultraviolet detector (Waters 2487 Dual Absorbance Detector) at 292 nm and quantified by comparing with internal standards. Se in plasma was determined by fluoroimetry with 2,3-diaminonaphthalene according to the method of Watkinson with modifications (31).

Histopathological analysis

At the end of Week 25, rats were killed 2 h after injection of 5-bromo-2-deoxyuridine (50 mg/kg, intraperitoneally) and the esophagi were excised and opened longitudinally. Tumors ≥1 mm in diameter were counted. The esophagus was cut longitudinally, with one-half fixed in 10% neutral buffered formalin for pathological analysis. The other half of the esophagus was stripped of muscle, quickly frozen in liquid nitrogen and then stored at −80°C for western blotting and enzyme immunoassay (EIA). Serial 5 μm esophageal sections were cut for staining. Hematoxylin and eosin stained sections were used for histological diagnosis, normal, hyperplasia, dysplasia, papilloma and ESCC. Diagnostic criteria were the same as those of Pozharasski (32).

Immunohistochemical staining

5-Bromo-2-deoxyuridine immunohistochemistry was performed using an in situ detection kit (BD Pharmingen, San Jose, CA) according to the manufacturer’s instruction. The proliferation index was calculated by dividing the number of positively stained cells by the total number of epithelial cells. 8-Hydroxy-2′-deoxyguanosine (8OH-dG) was analyzed similarly. In brief, antigens were unmasked in citrate buffer for 10 min at 95°C. Endogenous peroxidase was quenched by a 10 min incubation in 3% hydrogen peroxide in phosphate-buffered saline. Sections were incubated with primary antibody (1:250; Millipore, Billerica, MA) overnight at 4°C. Negative controls were processed in the absence of the primary antibody. Horseradish peroxidase-conjugated secondary antibody (Zhongshanjinqiao, Beijing, China) and diaminobenzidine were then applied to the sections. Six non-contiguous,
randomly selected fields in each lesion type were photographed under ×400 magnification. Stain-positive cells were analyzed by Image-Pro system.

Microvessels were detected by immunofluorescence staining for von Willebrand factor, a marker for vascular endothelial cells. After antigen retrieval with citrate buffer, the sections were incubated with either a rabbit anti-cyclooxygenase 2 (COX2) antibody or a rabbit 5-lipoxygenase (5LOX) antibody (Abcam, HongKong, China) at 1:500 overnight at 4°C and then with a horseradish peroxidase-labeled secondary antibody (Santa Cruz, Santa Cruz, CA) at 1:2000 for 2 h at room temperature. β-Actin was detected in the same samples as the loading control. Densitometric analyses of the immunobLOTS were performed using the GeI Imaging System and the Quantity One Software version 4.0 (Bio-Rad).

**Statistical analysis**

Incidence rates of visible tumors, papilloma and carcinoma of the treatment groups were compared using the χ² test and Fisher’s exact test. Tumor multiplicity, number of microscopic lesions and blood sample markers were expressed as mean ± standard deviation and evaluated using one-way analysis of variance. Comparisons between groups were made using Tukey-Kramer test. All the analysis was performed using the SAS statistical computer program (SAS Institute, Cary, NC).

**Results**

**General observations and nutritional status of NMBzA-treated rats**

The general appearance and activity level of animals were not affected by treatments: continuous low Ve/Se diet (Group A), early stage supplementation (by switching from the low Ve/Se diet to the AIN-93M diet) (Group B), late stage supplementation (Group C) and continuous supplementation (Group D). Body weights within 10 weeks did not show any significant difference among the five groups. After Week 10, body weights of Groups A and C became statistically less than that of Groups D and E (P < 0.05). From Weeks 19 to 25, average body weights of all the NMBzA-treated groups remained lower than that of control group (Group E, P < 0.05). Food consumption in NMBzA-treated groups also gradually reduced after Week 14 (P < 0.05). However, Ve/Se supplementation at the early (Group B) or late stage (Group C) had no different effect on body weight.

As shown in Figure 1B and C, both plasma α-tocopherol and Se levels in NMBzA-treated groups decreased after carcinogen treatment at Week 5. The carcinogen treatment had a profound effect in lowering the plasma α-tocopherol level in the rats on the low Ve/Se diet; maintaining rats on this diet for 25 weeks produced a very low α-tocopherol level (1 μg/ml), but signs of deficiency were not observed. Compared with Group A (which consumed low Ve/Se diet), Ve/Se supplementation at the early stage (Groups B and D) resulted in significantly higher plasma levels of α-tocopherol and Se at Week 15 (P < 0.05). Switching to low Ve/Se diet during the late stage (Week 11–25) decreased α-tocopherol and Se levels in Group B after Week 15. In contrast, α-tocopherol and Se levels in late supplementation group (Group C) were elevated and became higher than those of Group B (P < 0.05) at Weeks 15 and 25.

**Esophageal carcinogenesis of NMBzA-treated rats**

To determine the dose of NMBzA and timing of the development of esophageal lesions for the present study, a preliminary experiment was performed and the results are presented as supplementary Table 1, available at Carcinogenesis Online. In summary, this preliminary experiment showed that 0.35 mg/kg body wt was more suitable for our study than 0.5 mg/kg. At 0.35 mg NMBzA/kg, hyperplasia was the most prevalent lesion observed in the esophagus (70% of the rats) 10 weeks after NMBzA treatment. At this time point, 20% of the rats had papillomas, but no carcinoma was observed. At week 15, however, 10% of the rats developed carcinoma. Therefore, we set Week 10 as the dividing point of the early and late stages to conduct our supplementation study.

The incidence and multiplicity of visible tumors are summarized in Table I. In comparison with Group A, which had a tumor incidence of 100%, early, late or continuous supplementation of Ve/Se (Groups B, C and D) significantly reduced tumor incidence to 82.9, 81.8 or 71.8%, respectively (P < 0.05). Continuous supplementation (Group D) appeared to be slightly more effective than early supplementation (Group B) and late supplementation (Group C), but the difference was not statistically significant. Tumor multiplicity was significantly decreased by early supplementation (Group B) and continuous supplementation (Group D) (P < 0.05), but not by late supplementation (Group C). In this experiment, 16 rats (three in Group A, five in Group B, seven in Group C and one in Group D) died prematurely from Weeks 22– to 24, possibly due to the obstruction of the esophagus by big papillomas. All these 16 rats had esophageal tumors, but the esophagus samples could not be used for histological analysis, therefore they were not included in Table I. If these dead rats (all with esophageal tumors were counted, the tumor incidence for Group A would be 100%, Group B would be 85%, Group C would be 85% and Group D would be 70.2%; the results of the statistical analysis still remained the same.

As for microscopic changes, the multiplicity of carcinoma was significantly reduced by early supplementation (Group B), late supplementation (Group C) and continuous supplementation (Group D), as compared with Group A (Table I). Furthermore, both early supplementation (Group B) and continuous supplementation (Group D) produced significantly less carcinomas than late supplementation (Group C) (P < 0.05). The incidence of ESCC was decreased in a similar pattern, but no significant difference was found between early supplementation (Group B) and late supplementation (Group C). Papilloma multiplicity was markedly decreased by early supplementation (Group B) and continuous supplementation (Group D) (P < 0.05), but not by late supplementation (Group C). When compared with Group A, the incidence of papilloma was significantly reduced by continuous supplementation (Group D) but not by early supplementation (Group B) and late supplementation (Group C). The number of dysplastic lesions was significantly decreased by early supplementation (Group B) and continuous supplementation (Group D) (P < 0.05), but not by late supplementation (Group C). No significant difference in the number of hyperplastic lesions was found among the four NMBzA-treated groups (Groups A, B, C and D), although all these groups produced more hyperplasia than the negative control group (Group E) (P < 0.05).

**Cell proliferation and angiogenesis in the esophagi of NMBzA-treated rats**

5-Bromo-2-deoxyuridine -labeled cells in normal esophageal basal layer were <10% but clearly increased in different histopathologic lesions (Figure 2). Quantitatively, the esophagi of Group A showed significantly more proliferative cells in lesions of hyperplasia (24%), papilloma (46%) and carcinoma (57%) than that of Group D (16, 24 and 42%, respectively) (P < 0.05). Proliferation indices in carcinoma of early and late supplementation groups (Groups B and C) were similar, and both were less than that of continuous low Ve/Se group (Group A) but higher than the continuous supplementation group (Group D) (P < 0.05). In lesions of dysplasia and papilloma, proliferation indices of early supplementation group (Group B, 23 and 30%) appeared to be less than those of late supplementation group (Group C, 31 and 39%), but without statistical significance (P > 0.05).
Table I. Prevention of NMBzA-induced esophageal carcinogenesis in rats by Ve/Se supplementation

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Incidence (%)</th>
<th>Multiplicity</th>
<th>Number of hyperplasia</th>
<th>Number of dysplasia</th>
<th>Papilloma incidence (%)</th>
<th>Papilloma multiplicity</th>
<th>Carcinoma incidence (%)</th>
<th>Carcinoma multiplicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (Continuous low Ve/Se diet)</td>
<td>37</td>
<td>100.0</td>
<td>2.46 ± 1.23a</td>
<td>2.84 ± 1.72</td>
<td>2.11 ± 1.12</td>
<td>73.01</td>
<td>1.05 ± 0.85</td>
<td>37.81</td>
<td>0.46 ± 0.69</td>
</tr>
<tr>
<td>B (Early supplementation)</td>
<td>35</td>
<td>82.9</td>
<td>1.77 ± 1.17b</td>
<td>2.86 ± 1.48</td>
<td>1.26 ± 1.26</td>
<td>54.31,2</td>
<td>0.57 ± 0.56</td>
<td>20.01,2</td>
<td>0.20 ± 0.41</td>
</tr>
<tr>
<td>C (Late supplementation)</td>
<td>33</td>
<td>81.8</td>
<td>1.91 ± 1.40a</td>
<td>3.10 ± 1.89</td>
<td>1.90 ± 1.16</td>
<td>51.61,2</td>
<td>0.68 ± 0.75</td>
<td>35.51</td>
<td>0.35 ± 0.49</td>
</tr>
<tr>
<td>D (Continuous supplementation)</td>
<td>39</td>
<td>71.8</td>
<td>1.49 ± 1.41b</td>
<td>3.31 ± 2.04</td>
<td>0.93 ± 1.03</td>
<td>27.62</td>
<td>0.34 ± 0.61</td>
<td>15.42</td>
<td>0.14 ± 0.35</td>
</tr>
<tr>
<td>E (Negative control)</td>
<td>30</td>
<td>0</td>
<td>0</td>
<td>0.30 ± 0.67b</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

The numbers of tumors and microscopic lesions (hyperplasia, dysplasia, papillomas and carcinoma) were expressed (mean ± SD) per animal. \(^1,2,3\)Values with different superscripts in each column are significantly different based on \(\chi^2\) test followed by Fisher’s exact test \((P < 0.05)\). \(ab, bc\)Values with different superscripts in each column are significantly different based on analysis of variance test followed by Tukey-Kramer test \((P < 0.05)\).

Fig. 2. Cell proliferation in rat esophageal epithelium as determined by immunohistochemical staining of 5-bromo-2-deoxyuridine: (A) normal epithelium, (B) hyperplasia, (C) low-grade dysplasia, (D) high-grade dysplasia, (E) papilloma and (F) ESCC from rats of Group A. (G) Proliferation index of each lesion was calculated as the number of 5-bromo-2-deoxyuridine-positive cells divided by the total number of epithelial cells. Esophageal tissues were randomly selected from 12 rats in each group and used for analysis \((n = 12)\). Six non-contiguous, randomly selected fields were photographed in each lesion type. The total cell numbers counted was 1263 ± 773 (mean ± standard deviation) per lesion type in different slides of the same group. Values with different superscripts in each column were significantly different based on analysis of variance test followed by Tukey-Kramer test \((P < 0.05)\).

In NMBzA-induced lesions, the neovasculatures mainly spread along stromal ridges in lamina propria (Figure 3). A few microvessels were observed in the epithelium especially in papilloma and carcinoma. The microvascular density of NMBzA-treated groups were significantly higher than that of negative control group (Group E) at each pathologic stage \((P < 0.05)\). Similar to cell proliferation, angiogenesis in papillomas and carcinomas of early supplementation group (Group B) appeared to be less than those of late supplementation group (Group C), but the difference was of no statistical significance \((P > 0.05)\).

Oxidative DNA damage in the esophagi of NMBzA-treated rats

In order to study DNA oxidative damage, the level of 8OH-dG was determined by immunohistochemistry and analyzed by Image-Pro system. As shown in Figure 4B–F, strong nuclear stains of 8OH-dG were observed in the epithelium especially in papilloma and carcinoma as compared with Group A \((P < 0.05)\). In dysplasia, early supplementation also reduced the percentage of 8OH-dG-positive cells (Group B, 32.7%), which was significantly less than that of late supplementation group (Group C, 44.1%). The 8OH-dG-positive cells in papilloma and carcinoma of early supplementation group (Group B) appeared to be lower than those of late supplementation group (Group C), but the differences were not statistically significant \((P > 0.05)\).

Aberrant arachidonic acid metabolism in the esophagi of NMBzA-treated rats

As shown in the western blots in Figure 5A and B, both COX2 and 5LOX were apparently overexpressed in NMBzA-treated groups (Groups A, B, C and D) in comparison with the negative control group (Group E). Densitometric analyses (Figure 5C and D) demonstrated that overexpression was highest in the continuous deficiency group (Group A) \((P < 0.05)\). Early supplementation (Group B) or late supplementation (Group C) both decreased the levels of COX2 and 5LOX \((P < 0.05)\). However, they were not significantly different from continuous supplementation (Group D) \((P > 0.05)\).

PGE2 and LTB4 levels in the esophageal tissues were similarly increased by NMBzA treatment in comparison with the negative control (Group E) (Figure 5E and F). They were significantly reduced by continuous supplementation (Group D) \((P < 0.05)\). When compared with late supplementation (Group C), early supplementation (Group B) appeared to be more effective in reducing PGE2/LTB4 production although without statistical significance \((P > 0.05)\).
Discussion

Our results demonstrated that low Ve/Se diet significantly enhanced esophageal carcinogenesis in NMBzA-treated rats. Supplementation of Ve/Se to the normal nutritional levels during the early stage, the late stage or throughout the entire experimental period inhibited carcinogenesis in this model. Based on some key parameters (visible tumor multiplicity, carcinoma number, numbers of dysplastic lesions and papilloma), early supplementation was more effective in suppressing carcinogenesis than late supplementation. These results are in agreement with those from intervention trials in Linxian, which suggest that the timing of the intervention is a factor in affecting the outcome (10,12,13,16,17).

In our model, NMBzA is known to be metabolized by cytochrome P450 enzymes to form methyl carbonium ions, which methylate DNA to produce O\textsubscript{6}-methylguanine adducts (33,34). The observations that p53 and H-ras gene mutations, mostly G:C→A:T transitions, are consistent with O\textsubscript{6}-methylguanine adduct formation during the
initiation and development of esophageal carcinogenesis induced by NMBzA (35). In addition, free radicals generated from metabolic activation of NMBzA or other processes may elicit oxidative DNA damage and produce extra mutations. Accumulation of endogenous reactive oxygen species caused by lower nutritional status in antioxidants contributes to further DNA oxidative damage as well as lipid peroxidation (36). As shown in this experiment, 8OH-dG was significantly increased in various lesions in the esophagi of NMBzA-treated rats. Many antioxidative micronutrient or phytochemicals were found to be protective against NMBzA-induced esophageal cancer (37–39). Some epidemiological and intervention studies and laboratory experiments have suggested the possible role of Ve/Se in suppressing cancer, because of their antioxidative functions (40,41). Our study also clearly showed that continuous supplementation of Ve/Se decreased 8OH-dG in each category of lesions, and this correlated well with the plasma levels of α-tocopherol and Se at Week 25.

During carcinogenesis, inflammation and oxidative stress promoted each other in a vicious circle (42,43). Key mediators that link inflammation to cancer via oxidative stress are metabolites of arachidonic acid (AA) such as PGE2 and LTB4 (43). Recent research has clearly demonstrated that aberrant AA metabolism is involved in human carcinogenesis, including esophageal cancer (44). As a critical enzyme in AA metabolism, COX2 overexpression was related to cell proliferation in esophageal dysplasia and ESCC (45,46). Similarly, 5LOX overexpression also promoted tumorigenesis, and 5LOX pathway inhibitors showed chemopreventive effect in animal models of esophageal cancer (47). In the present study, we observed that continuous supplementation of Ve/Se clearly decreased COX2 and 5LOX protein expression. PGE2 and LTB4, the respective metabolites of COX2 and 5LOX, were reduced as well. PGE2 stimulates cell proliferation in a variety of carcinoma cell types in vitro and enhances angiogenesis in vivo (48). COX2 inhibitors have been reported to suppress cell proliferation by blocking PGE2 production. It is suggested that tumor growth may be supported by PGE2 through inducing angiogenesis, which is necessary to supply oxygen and nutrients to tumors. In agreement with these findings, our study demonstrated that cell proliferation and angiogenesis were significantly inhibited by continuous supplementation (Group D) with
suppressed expression of COX2/SLOX as well as production of PGE2/LTB4. These data suggested that COX and LOX pathway of AA metabolism may be important in NMBzA-induced carcinogenesis in rat esophagus.

More importantly, we discovered that early supplementation had stronger preventive effect than late supplementation. Compared with late supplementation, the early supplementation group had generated significantly less carcinoma and dysplasia. The numbers of papilloma and the incidence of carcinoma also appeared lower in the early supplementation group. In addition, early supplementation produced significantly less DNA oxidative damage in dysplasia. Although we did not observe time-dependent changes of DNA oxidative damage in the esophagus, DNA damage and aberrant AA metabolism initiated by NMBzA could be effectively prevented by supplementation with Ve/Se at the early stage. Once the primary lesions are established, Ve/Se may be less effective in reducing oxidative damage and aberrant AA metabolism, or enhancing the growth of lesions, and became less effective in preventing carcinogenesis.

Baseline nutrients levels are probably to modify an individual’s response to nutrient supplementation. Results from the Linxian trials strongly suggested that supplementation was more probably to be beneficial for individuals who were low or borderline deficient in micronutrients (49). In the SELECT trial, Ve and Se failed to prevent prostate cancer in individuals that were well nourished in Ve and Se (19). However, if the trial were conducted with subjects with low nutritional status with Ve and Se, the supplementation might have been found to prevent prostate cancer. As one of the limitations in the present animal study, the supplementation was performed only at normal dietary levels. Although these levels were effective, further dose-dependent studies will help us to understand the cancer prevention process better.

In summary, our study demonstrated that NMBzA-induced esophageal carcinogenesis in rats on a low Ve/Se nutritional status could be prevented by dietary supplementation of Ve/Se. Suppression of cell proliferation and angiogenesis by inhibiting oxidative stress and aberrant AA metabolism might be the underlying mechanism. Furthermore, Ve/Se supplementation at the early stage of esophageal carcinogenesis had a relatively stronger effect than that at the late stage. Our data provide experimental support to the conclusion of the clinical trial that Ve/Se supplementation decreased esophageal cancer deaths among younger participants but not in older participants who were more probably to have precancer or severe precancerous lesions. This present study in an animal model and the human studies (10–15) support the hypothesis that marginal deficiencies or lower status in micronutrients enhance the risk for esophageal cancer caused by environmental carcinogens such as dietary nitrosamines or their precursor, as well as genetic susceptibility factors (50).

Supplementary material
Supplementary Table 1 can be found at http://carcin.oxfordjournals.org/.

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References
33. Siglin, J.C. et al. (1996) O6-methylguanine levels and histopathological changes in the rat oesophagus and liver following single and repeated administration of N-nitrosomethylbenzylamine. Carcinogenesis, 17, 1135–1140.

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