



ADMINISTRATION OF VITAMIN E PREVENTS THYMOCYTE APOPTOSIS IN MURINE SARCOMA S180 TUMOR BEARING MICE

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Abstract

Many reports claimed that thymic involution occurred in tumor bearing host, which led to a reduction of proliferation in T cells and an impaired immune system. In this study, murine sarcoma S180 tumor cells were used to establish tumor model. There was distinct apoptosis in thymus of the S180 tumor bearing host after 3-week tumor inoculation. High level reactive oxygen species generation was detected in thymocytes of S180 tumor bearers. Vitamin E, a potent antioxidant, was oral administrated to S180 tumor bearing mice in order to eliminate redundant ROS in the host and evaluate the effect against thymic apoptosis. Intact thymic structure and less apoptosis of thymocytes demonstrated that partial restoration of thymuses in S180 tumor bearing mice after 3-week VE treatment. Besides, VE treatment normalized ROS level, MDA level and SOD level of S180 tumor bearers. Thus, it could be concluded that thymic involution prevented by VE treatment in S180 tumor bearing mice was mainly due to inhibition of apoptosis in thymus and elimination of ROS over-production in the host.

Key words: Vitamin E, reactive oxygen species, S180 tumor bearing mice, thymic involution, apoptosis.

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INTRODUCTION

The thymus is the major site of T lymphocytes proliferation, differentiation and maturation (21). It has a complex cellular composition, which is mainly constituted by cortex and medulla (3, 39). The development of T cell is characterized by the progression through several phenotypically distinct stages (19). These processes are important for the positive selection of immature thymocytes and the deletion of self-reactive T cell clones, respectively (30). In this case, mature and sufficient T cells can struggle to eliminate tumors effectively. However, a host of reports have been claimed that inefficiency of tumor-specific T cells indulges tumor development (10, 24, 28), which may be a link with thymic atrophy (1, 23).

In recent years, acute thymic atrophy has been extensively observed in many model systems, such as graft-vs-host disease (38), stress (7), infections (26), acute inflammation (34) and tumor development (1). It is well known that peripheral T cell functions of the tumor-bearing mammals, including humans, are greatly impaired due to thymic involution (14). This thymic involution is mainly caused by apoptotic cell death in thymocytes, including a down-regulation of anti-apoptotic molecules such as Bcl-X_L and A1 (5). Moreover, down-regulation of crucial cytokines such as interleukin-7 leads to deficiency in thymocyte population, destruction in thymic microenvironment and prolonged T cell development in tumor-bearing mammals and humans (6). However, the mechanisms involved in this phenomenon remain to be complete elucidated.

Reactive oxygen species (ROS) play an essential role in tumorigenesis and tumor cell metastasis (15). Most tumor cells exhibit elevated oxidative stress and production of ROS (35, 17). High ROS levels may damage organs, even

induce growth arrest or apoptosis (29). Thymus is susceptible to these free radicals, leading to apoptotic thymocyte death and degeneration of this organ (33). In this study, Kunming (KM) mice were used for establishing S180 tumor model. Vitamin E (VE), served as a potent antioxidant, was oral administered to S180 tumor bearing mice for 21 consecutive days. According to the reports on the adequate dose of VE for mice (25, 31), 80 IU/kg was the optimum dose in this study. Histological stain was used to illustrate the protective effect on thymic involution of S180 tumor bearers after VE treatment. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay confirmed that few apoptotic thymocytes were detected in the thymus from VE treated group. Expression levels of Bcl-2, Bcl-X_L and Caspase 3 in thymus from VE treated group were also normalized as compared with the model group. The intracellular generation of ROS in thymus was inspected by 2' 7'-Dichlorofluorescein diacetate (DCFH-DA) probe. Moreover, malondialdehyde (MDA) level, superoxide dismutase (SOD) level in both serum and thymus of each group were also determined.

MATERIALS AND METHODS

Materials

VE was purchased from Sigma-Aldrich Chemical Company (St Louis, MO, USA). The molecular probe of DCFH-DA was purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). TUNEL apoptosis detection kit was purchased from Promega (Madison, WI, USA). The primary antibodies used were anti-Bcl-X_L, anti-Bcl-2 and anti-Caspase-3 all from Millipore (CA, USA). Antibodies against β -actin and horseradish peroxidase conjugated secondary antibodies were obtained from

Santa Cruz Biotechnology (CA, USA). Concanavalin A (ConA) and methyl thiazolyl tetrazolium (MTT) were both purchased from Sigma-Aldrich (MO, USA). MDA Colorimetric Assay Kit and SOD Colorimetric Assay Kit were all obtained from Nanjing Jiancheng Bioengineering Institute (Jiangsu, China). All other chemicals were of the highest commercial grade available.

Mice and cell line

Male and female KM mice, body weight 18-22 g, were purchased from the Department of Experimental Animals, Academy of Military Medical Science, Beijing, China. All the mice were under barrier conditions in the Centre of Experimental Animals at Tianjin University of Science and Technology. The animals were allowed free access to food and water at all times and were maintained on a 12 h light/dark cycle in a controlled temperature (20-25 °C) and humidity (50±5%) environment. The National Institutes of Health guidelines for the care and use of laboratory animals were followed in all animal experimental procedures. S180 cells (murine sarcoma S180 tumor cell line) and NIH-3T3 cells (murine embryonic fibroblast cell line), purchased from Shanghai Institute for Biological Sciences (Chinese Academy of Sciences, Shanghai, China), were cultured in RPMI-1640 containing 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin G, 100 mg/ml streptomycin, and 2 mmol/l glutamine.

Cell proliferation assay

S180 cells and NIH-3T3 cells were transferred to 96-well plates containing RPMI-1640 medium supplemented with 10% fetal bovine serum. The optimal cell number was found to be 2×10^4 cells per well by bacteria count. The cells were allowed to settle overnight and were treated with varying concentrations of VE (0, 25, 50, 100 µg/mL) with 0.5% ethanol for 24 h at 37 °C in a humidified 5% CO₂ incubator. After incubation, MTT solution (0.5 mg/mL in PBS) was added for 4 h at 37 °C. The precipitated formazan was dissolved in 150 µL dimethyl sulphoxide (DMSO) and the plates were read at 570 nm and reference absorbance at 630 nm with a microplate reader (Model 680, Bio-Rad, Hercules, Calif., U.S.A.). All experiments were carried out in triplicate.

Cell survival rate (%) = (absorbance of cells treated with VE / absorbance of control cells) × 100%.

VE treatment

Ninety mice were randomly divided into control group (30 mice), S180 group (30 mice) and VE treated group (30 mice). On the first day, S180 group and VE treated group were subcutaneous injected with S180 sarcoma cells (1.5×10^6 cells/mouse) to set up S180 tumor model. VE treated group was oral administered with 80 IU/kg VE everyday, while control group and S180 group were treated with normal saline 0.2 ml/mouse everyday. After 3 weeks, blood of mice from different groups was collected into eppendorf tubes, respectively. After centrifugation, serum was collected and stored at -80 °C for further studies. The average survival time of each group was recorded and the increase in lifespan was calculated: Increase in lifespan (ILS) = (average lifespan of VE group - average lifespan of S180 group) / average lifespan of S180 group × 100%.

Thymus collection and histological staining by H-E

Thymus was carefully dissected from the chest cavity and placed in a petri dish containing sterile normal saline. The thymic lobes were weighed and placed in a petri dish with 10% neutral-buffered formalin for more than 3 h, and dipped in running water overnight, and gradually dehydrated in ethanol of different strengths, dimethylbenzene, and embedded in paraffin, and processed for histological examination after staining with hematoxylin-eosin.

T lymphocyte transformation test

Spleen cells (2×10^6 /ml) of each group were suspended in RPMI 1640 containing 10% FBS, separately. The wells were filled by adding 100 µl of spleen cells and 10 µg/ml ConA. The cells were incubated for 68 h at 37 °C in a humidified 5% CO₂ incubator. After incubation, MTT solution (0.5 mg/ml in PBS) was added for 4 h incubation at 37 °C. The precipitated formazan was dissolved in 150 µl DMSO and the plates were read at 570 and 630 nm using a microplate reader (Model 680, Bio-Rad, Hercules, Calif., U.S.A.). All experiments were carried out in triplicate. Stimulus index (SI) = experimental OD570-630/control OD570-630.

TUNEL assay

The slide of thymus was treated with 20 µg/ml DNase-free proteinase k at room temperature for 30 min. TUNEL staining was performed using death detection kit according to the manufacturer's instructions. Specimens were examined and photographed under a laser scanning confocal fluorescence microscope (Nikon, 90i, Tokyo, Japan) with excitation at 488 nm and emission at 525 nm.

Western blot

The whole cell extracts from thymuses of different groups were used. The cells were washed with ice-cold PBS, drained the PBS and added ice-cold RIPA buffer to extract whole cell protein, which contained 50 mM Tris-HCL (pH 7.4), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS and 0.1 mM PMSF, maintained for 30 minutes at 4 °C and centrifuged 20 minutes at 12000 rpm at 4 °C. Performed a Bradford assay to determine the protein concentration levels. 12% PAGE gel were prepared and the samples were subjected to gel electrophoresis at a constant current of 20 mA per gel. After finishing the SDS-PAGE, the proteins were transferred onto nitrocellulose membrane in wet condition. The membrane was blocked by non-fat milk for 1 h at 4 °C to prevent non-specific background binding of the primary and/or secondary antibodies to the membrane. The primary antibodies used were anti-Bcl-X_L, anti-Bcl-2 and anti-Caspase-3 as described above and the diluted primary antibodies (1:350) incubated the membrane at 4 °C overnight. The secondary antibodies, a HRP-conjugated goat anti-mouse IgG diluted at 1:1000, incubated the membrane for 1 h at room temperature. X-ray films were used for manual development and analyzed with Quantity One software.

Measurement of ROS production

The intracellular generation of ROS was inspected by DCFH-DA. The nonfluorescent ester penetrated into the cells and was hydrolyzed to DCFH by the cellular esterases. The probe is rapidly oxidized to the highly fluorescent compound 2',7'-dichlorofluorescein (DCFH) in the

presence of cellular peroxidase and ROS such as hydrogen peroxide or fatty acid peroxides. Briefly, fresh thymus was isolated into a uniform single-cell suspension by using a cell strainer (BD, USA). Then, cells (1×10^6) were centrifuged and loaded with $10 \mu\text{M}$ DCFH-DA for 30 min at room temperature in the dark, then washed with PBS (pH 7.2) three times. The fluorescence was measured at an excitation of 488 nm and an emission of 525 nm.

Determination of MDA level and SOD level in serum and thymuses

Thymus (100 mg, wet weight) was homogenized in 2 ml of 10 mM ice-cold phosphate buffer (pH 7.4). After centrifugation at 12000 g for 20 min, the supernatant was collected for the following experiment. MDA and SOD content in serum and thymus were determined with chemical method according to the manufacturer's instructions. MDA content was measured with thiobarbituric acid (TBA) reaction. The method was used to obtain a spectrophotometric measurement of the color produced during the reaction of TBA with MDA at 535 nm. MDA level in serum was expressed as nmol/ml, while MDA level in thymus was expressed as nmol/mg-protein. SOD activity was measured using nitroblue tetrazolium chloride (NBT) reduction assay following the reduction of nitrite by a xanthine-xanthine oxidase system, which is a superoxide anion generator. SOD level in serum was expressed as U/ml, while SOD level in thymus was expressed as U/mg-protein.

Statistical analysis

Experiments were performed at least three times with similar results. Results were expressed as the mean \pm standard deviation of the indicated number of replicates, and differences between groups were assessed using SPSS software (Statistical Product and Service Solutions USA). To analyze the data statistically, analysis of variance (ANOVA) was performed for multiple comparison. Fisher's protected least significant difference (Fisher's PLSD) was used for identifying significant differences. For correlation, simple regression or multivariate analysis was used. A P value of less than 0.05 was considered to have statistical significance for all results in this study.

RESULTS

VE inhibited S180 tumor cell proliferation in vitro

Cell survival rate of S180 cells and NIH-3T3 cells treated with VE for 24 h were measured by MTT assay as described in materials and methods. As shown in Fig. 1, the results indicated that VE had a dramatic suppression on S180 cells proliferation. At the concentration of $50 \mu\text{g/mL}$, the cell survival rate of S180 at 24 h was about 50%. In contrast, VE had less effect on NIH-3T3 cells for 24 h treatment. Even at the concentration of $100 \mu\text{g/mL}$, the cell survival rate of NIH-3T3 at 24 h was above 80%. These results indicated that VE treatment in vitro specially inhibited S180 cells, whereas it had low cytotoxicity on the somatic cells, such as NIH-3T3 cells.

VE treatment prevents thymic involution and increases life span of S180 tumor bearers

To determine the protective effect of VE treatment on S180 tumor bearing mice, indexes of body weight, thy-

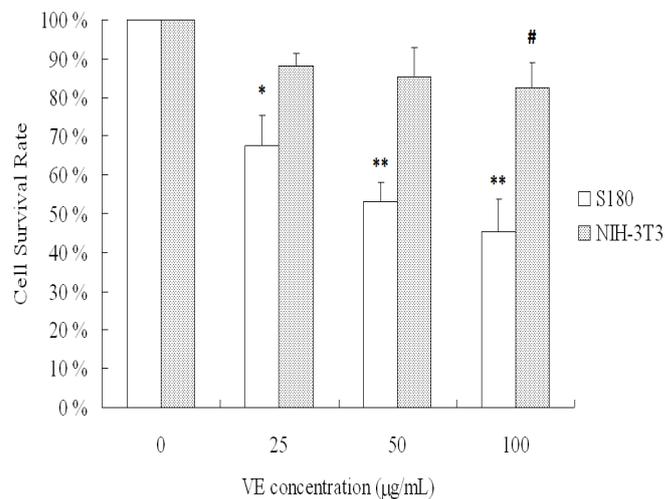


Figure 1. Cell survival rate of S180 cells and NIH-3T3 cells treated with VE for 24 h. Cells were treated with VE at the various concentration (0, 25, 50, 100 $\mu\text{g/mL}$). The cell survival rate was determined by MTT assay as described in the materials and methods. Data represent means \pm SD of three independent experiments. * $P < 0.05$ compared with S180 cells with 0 $\mu\text{g/mL}$ VE. ** $P < 0.01$ compared with S180 cells with 0 $\mu\text{g/mL}$ VE. # $P < 0.05$ compared with NIH-3T3 cells with 0 $\mu\text{g/mL}$ VE.

mus weight, tumor volume and weight, peripheral T cell transformation and increasing in life span were recorded. During the 21-day experiment, the body weight of model group grew faster (Fig. 2(a)), and thymus weight of S180 tumor bearing mice was dramatically declined (Fig. 2(b)). The tumor volume and weight of the model group increased remarkably in a time-dependent manner (Fig. 2(c, d)). In contrast, tumor growth of 3-week VE treated group was notably suppressed both in tumor volume and weight (Fig. 2(c, d)). The body weight and thymus weight of VE treated group became normalized as compared with model group.

T cells transformation test showed that stimulation index of T cells in S180 tumor bearing mice was intensely suppressed as tumor grown for 2 to 3 weeks, whereas stimulation index of T cells of 3-week VE treated group showed partial restoration as compared with control group (Fig. 2(e)). The data of 1-week and 2-week VE treatment were similar to those of 3-week VE treatment (data not shown). Besides, the survival time of 3-week VE treated group was notably increased than that of model group (Table 1). Collectively, these results suggested that VE treatment on S180 tumor bearing mice could prevent thymic involution, partial regain the immune function and increase life span of the S180 tumor bearers.

Histological analyses of thymuses from S180 tumor bearing mice and the effect of treatment with VE

After 3-week experiment, thymuses of different groups were collected and stained by H-E as described in materials and methods. As shown in Fig. 3, the histological analyses of thymuses from normal mice manifested a clear border between cortex and medulla, whereas the thymuses from 3 weeks S180 tumor bearing mice showed a shrunken medullar area and an indistinct medullar boundary at $\times 4$ magnification. The medullar area of the S180 tumor bearing mice was gradually dwindled from week 1 to week 3. Meanwhile, apoptotic bodies were first appeared in thymuses of 1 week S180 tumor bearers at magnification of $\times 100$, and the apoptotic cells became more in thymuses

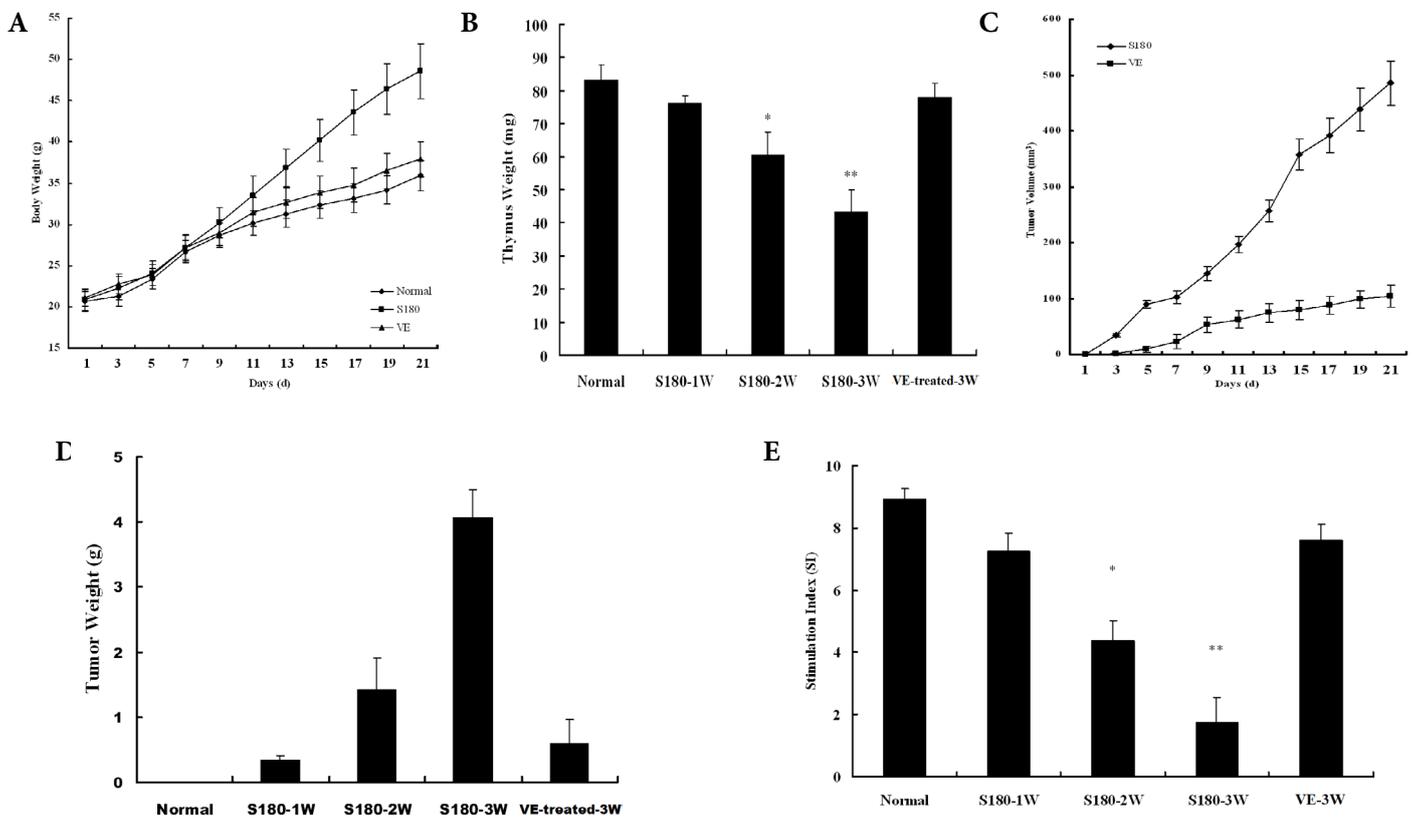


Figure 2. 3-week VE treatment prevents thymic involution and increases life span of S180 tumor bearers. (a), body weights of each group were determined every 2 days during the experiment. (b), thymuses of each group were carefully collected and weighted after the experiment. (c), tumor volumes of S180 tumor bearers and VE treated group were measured during the 21-day experiment. (d), solid tumors from each group were weighted after the 3-week experiment. (e), stimulation Indexes of T cells from each group were determined by lymphocyte transformation test. * P < 0.05 compared with control group. ** P < 0.01 compared with control group.

Table 1. The survival time and increased in lifespan of model group and VE treated group.

Group	Survival time (days)	Increased in lifespan
S180 tumor bearing mice	23.88 ± 1.89	-
VE treated mice	44.50 ± 5.40**	86.39%

** P < 0.01 compared with S180 tumor bearing mice group.

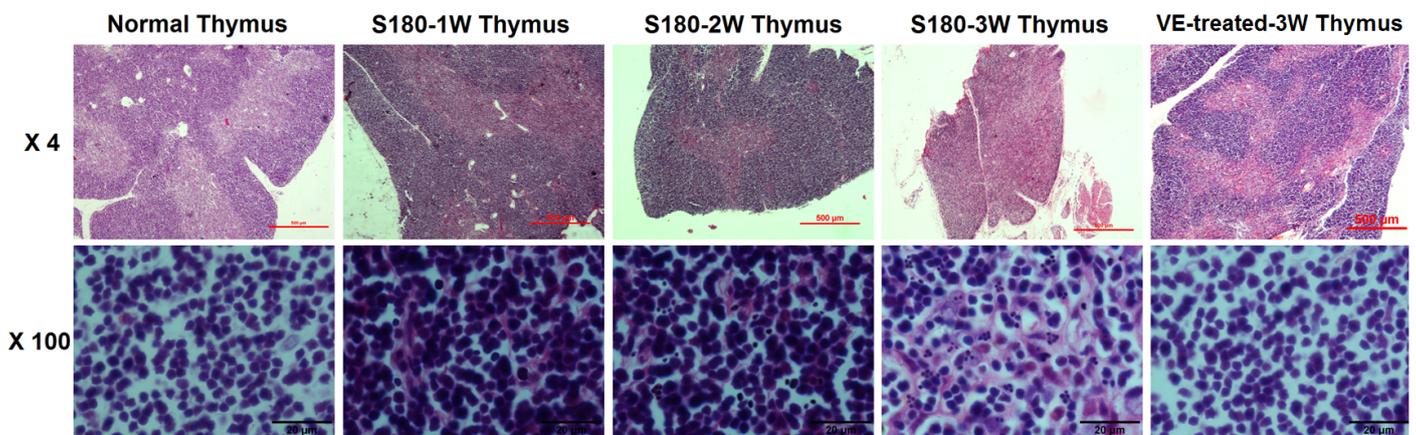


Figure 3. Histological analysis of thymuses from control group, model group and 3-week VE treated group. Bar length in the ×4 pictures was 500 µm, while in the ×100 pictures was 20 µm.

of 3 weeks S180 tumor bearers. However, thymuses of 3-week VE treated group illustrated a normalized structure, and no obvious apoptotic body was detected.

Apoptosis determined by TUNEL

As shown in Fig. 4, apoptotic cells were showed up in

thymuses of model group after 1 week S180 tumor implantation. As time went on, more apoptotic cells appeared in thymuses of S180 tumor bearers as compared with the control group after 2 and 3 weeks experiment. During the 3-week experiment, apoptosis was hardly detected in thymuses of VE treated group. It indicated that VE treatment

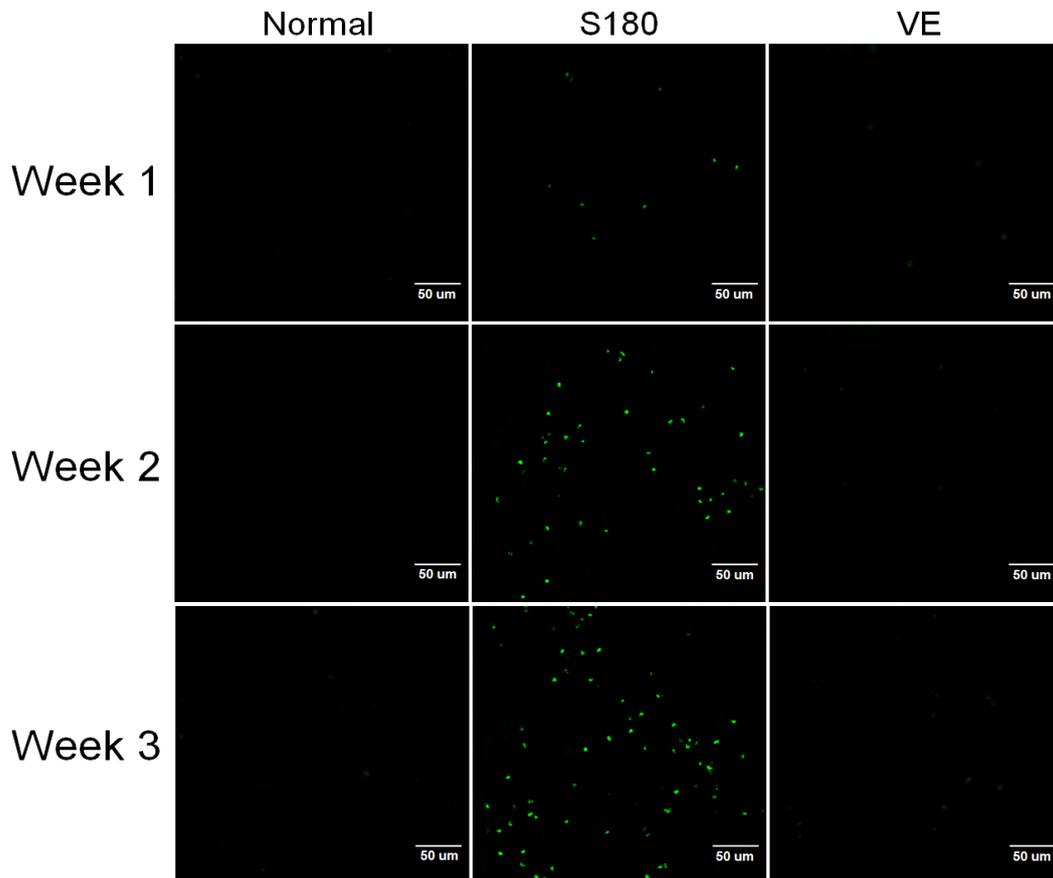


Figure 4. Detection of apoptosis in thymuses from different groups by TUNEL. Thymic sections of each group were surveyed after 1-week, 2-week and 3-week experiment. Bar length in the pictures was 50 μm . All pictures were analyzed at $\times 40$ magnification.

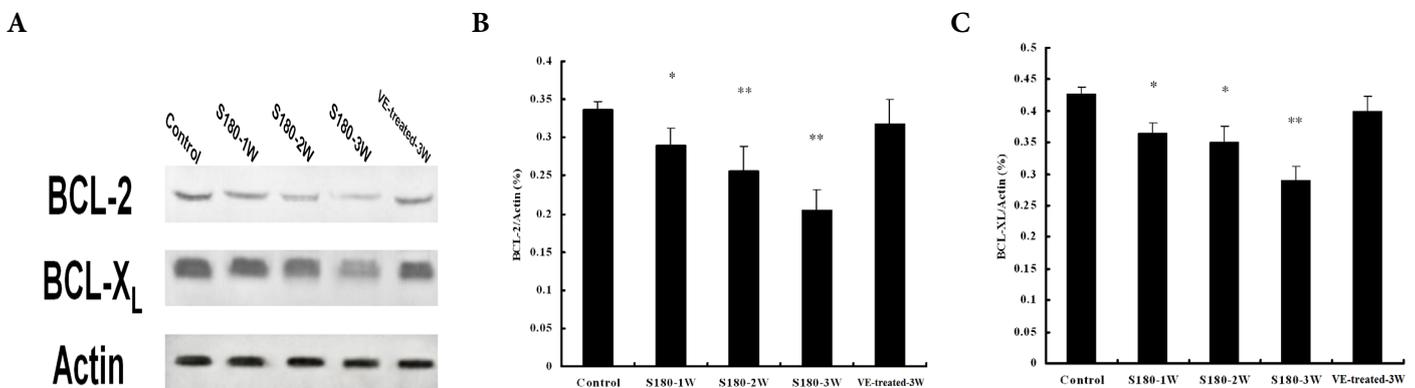


Figure 5. Expressions of Bcl-2 and Bcl-X_L in thymuses from each group. (a), western blot analysis of Bcl-2 and Bcl-X_L expressions in thymuses from different groups. (b), expressions of Bcl-2 in thymuses from different groups were subjected to densitometric analysis and normalized to Actin expression, respectively. (c), expressions of Bcl-X_L in thymuses from different groups were subjected to densitometric analysis and normalized to Actin expression, respectively. * $P < 0.05$ compared with control group. ** $P < 0.01$ compared with control group.

could prevent tumor-associated cell death in thymocyte of S180 tumor bearing mice.

Expression levels of Bcl-2, Bcl-X_L and caspase-3 in thymuses

In order to further investigate the apoptosis in thymuses during tumor development, expression levels of Bcl-2 and Bcl-X_L, inhibitors of apoptosis, were analyzed by western blot. Meanwhile, expression of caspase-3, executor of apoptosis, was also analyzed. As Bcl-2 and Bcl-X_L were highly regulated in thymus (9, 22), our data showed that expressions of Bcl-2 and Bcl-X_L were both declined in thymuses of tumor bearers as compared with control group (Fig. 5). However, expressions of Bcl-2 and Bcl-

X_L showed partial restoration in thymuses of S180 tumor bearing mice after 3-week VE treatment.

It's reported that pro-caspase-3 was activated by cleavage to generate a large subunit of 17-kDa and a small subunit of 12-kDa (32). As shown in Fig. 6, pro-caspase-3 was activated in thymuses of S180 tumor bearers. As time went on, the expression of cleaved caspase-3 was dramatically up-regulated in thymuses of model group from 1 to 3 weeks experiment. In contrast, caspase-3 activation in thymuses of VE treated group was inhibited after 3-week treatment. These data showed that 3-week oral administration of VE on S180 tumor bearing mice could prevent tumor-associated apoptotic cell death in thymocyte via up-regulation of both Bcl-2 and Bcl-X_L and inhibition of

caspase-3 activation.

Measurement of ROS production, MDA level and SOD level

To investigate ROS generation in thymocytes, thymuses of each group from 1 to 3 weeks were collected, respectively. As shown in Fig. 7, the DCFH fluorescence of thymocytes from model group gradually increased in a time-dependent manner during the 3-week experiment. However, after VE treatment on S180 tumor bearing mice, there was no significantly fluorescence intensity as compared with the model group. These data confirmed that thymocytes of S180 tumor bearers had higher ROS generation than the normal ones, but VE treatment could reduce this high ROS level in thymocytes of S180 tumor bearing mice.

Correspondingly, our data showed that MDA level was increased in serum and thymus of S180 tumor bearing mice as compared with the control group, but SOD level of S180 tumor bearers was dramatically decreased (Fig. 8(a, b)). After 3-week oral administration of VE to S180 tumor bearing mice, MDA level and SOD level were both normalized in both serum and thymus. It suggested that 3-week VE treatment on S180 tumor bearing mice inhibited tumor-associated high ROS environment in the tumor host.

DISCUSSION

The accumulated evidence indicated that many tumors induce T-cell apoptosis as a mechanism of inhibiting anti-tumor activity (8, 18, 37). This immune function impair-

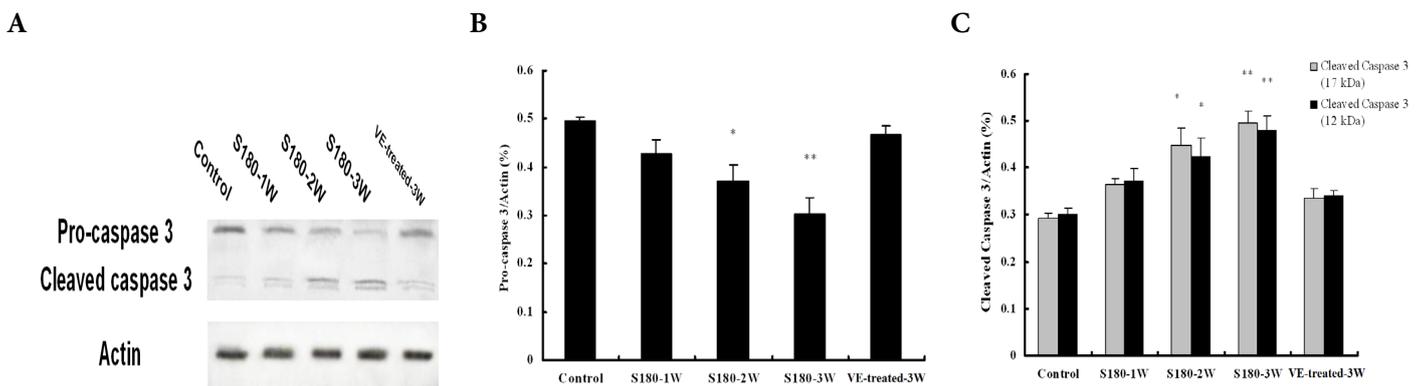


Figure 6. Expressions of caspase-3 in thymuses from each group. (a), western blot analysis of pro-caspase-3 and cleaved caspase-3 expressions in thymuses from different groups. (b), expressions of pro-caspase-3 in thymuses from different groups were subjected to densitometric analysis and normalized to Actin expression, respectively. (c), expressions of cleaved caspase-3 in thymuses from different groups were subjected to densitometric analysis and normalized to Actin expression, respectively. * $P < 0.05$ compared with control group. ** $P < 0.01$ compared with control group.

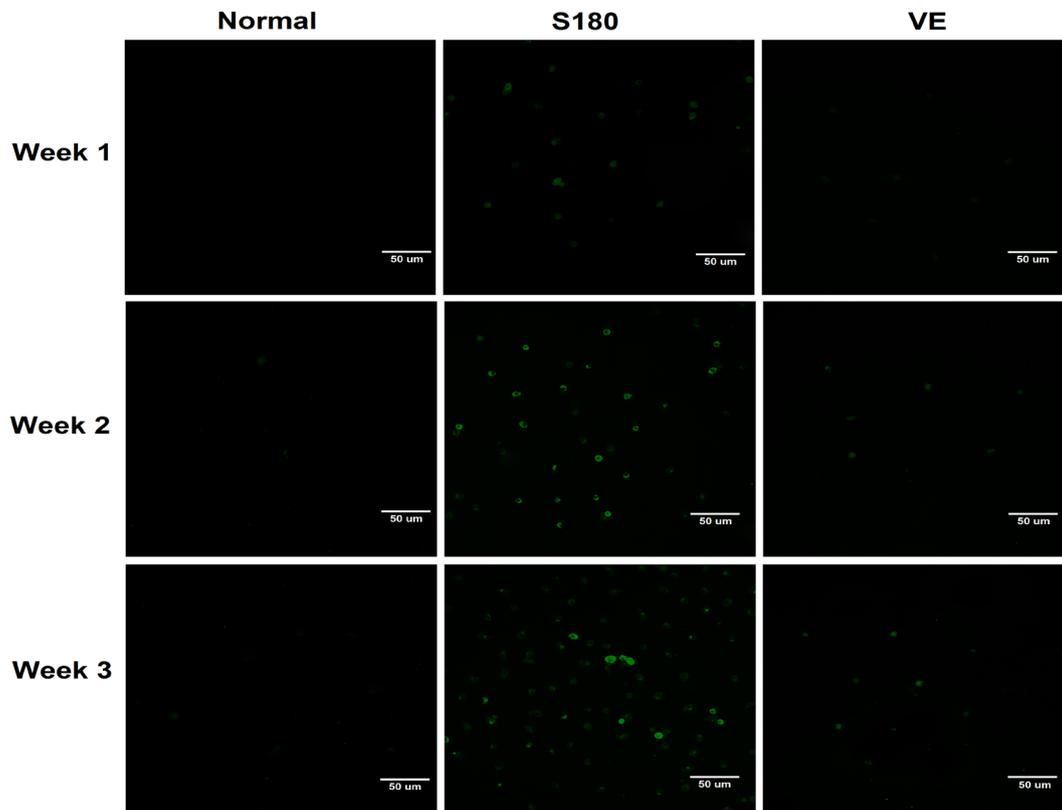


Figure 7. ROS generation in thymuses from different groups was inspected by DCFH-DA. Thymocytes of each group were surveyed after 1-week, 2-week and 3-week experiment. Bar length in the pictures was 50 μm . All pictures were analyzed at $\times 40$ magnification.

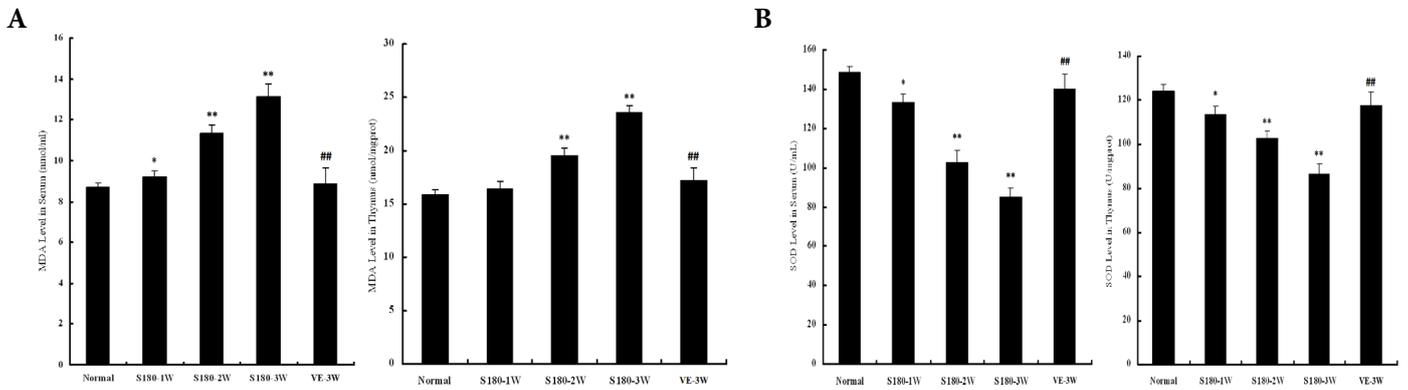


Figure 8. Determination of MDA level and SOD level in serum and thymuses from different groups. (a), MDA level in serum and thymus. MDA level in serum was expressed as nmol/ml, while MDA level in thymus was expressed as nmol/mg-protein. (b), SOD level in serum and thymus. SOD level in serum was expressed as U/ml, while SOD level in thymus was expressed as U/mg-protein. * $P < 0.05$ compared with control group. ** $P < 0.01$ compared with control group. ## $P < 0.01$ compared with 3-week S180 group.

ment might be causally related to the thymic involution, because the thymus is the major site of T-cell maturation, extensive proliferation and differentiation (3, 19, 21, 39). Recent reports indicated that tumor cells exhibited more oxidative stress than normal cells (35, 36). This high level ROS environment was important for tumor development and metastasis *in vivo* (15, 17), however, might be harmful to vulnerable organs such as thymus (33), leading to thymic involution and apoptosis (5, 6, 14). In this work, oral administration of VE to S180 tumor bearing mice showed a prolonged survival and a protective effect against thymic involution. The data indicated that VE could inhibit proliferation of S180 cells both *in vitro* and *in vivo*. Moreover, normalized histological structure of thymus and restoration of T cell function were detected in S180 tumor bearers after VE treatment. These results demonstrated that VE treatment indeed had an antitumor effect on S180 tumor bearing mice and prevented thymic involution. The detailed mechanisms involved in this phenomenon are elaborated further below.

Oxygen free radicals have the capacity to damage cellular components such as proteins, lipids, and nucleic acids. The ROS-related apoptotic cell death has been detected in many cell lines (2, 4, 41), and high oxidative stress has been detected in tumor models (20). One of the common features associated with cancer cells is increased ROS generation. Most cancer cells exhibit elevated oxidative stress with increased metabolic activity and over-production of ROS (35). It reports that ROS serve as signaling molecules to regulate tumor cell proliferation and cell cycle progression (11). In this work, as the solid tumor developing, increasing level of MDA and decreasing level of SOD were detected in both serum and thymus of S180 tumor bearing mice. Over-production of ROS in thymus of S180 tumor bearers gradually increased in a time-dependent manner during 21-day experiment. Subsequently, constant ROS over-production might induce the mitochondrial-dependent apoptosis (12, 27), and more apoptotic thymocytes were detected by TUNEL assay. It's known that mitochondrial-dependent apoptosis was executed by intrinsic pathway, which down-regulated anti-apoptotic proteins such as Bcl-2 family members and activated caspase cascade to induce cell apoptosis (40). In this case, high apoptosis in thymus would cause deficiency or inefficiency of peripheral T cells, resulting in uncontrollable tumor progression.

As cancer cells are sensitive to oxidative stress, modulation of SOD has been exploited as a mechanism to selectively kill cancer cells (13). Other antioxidant such as tocopheryl succinate is reported to selectively kill cancer cells (16). Our data confirmed that VE effectively inhibited S180 tumor cells proliferation and did less harm to normal cells, such as NIH-3T3 cells. Besides, VE treatment on S180 tumor bearing mice dramatically reduced the oxidative stress in the hosts, which would prevented ROS over-production and apoptosis in thymuses. As thymic microenvironment is vulnerable, increasing ROS level in S180 tumor bearers will disrupt thymic homeostasis. However, the result of VE treatment on S180 tumor bearers showed a protective effect against thymic involution, and normalized the immune system.

Based on our data, it could be concluded that thymic involution prevented by VE treatment in S180 tumor bearing mice was mainly due to inhibition of apoptosis in thymus and elimination of ROS over-production in the host. Whether high ROS level served as an apoptotic signal in thymocytes, it needed to be proved in our further studies. On the other hand, the formation of high ROS environment in tumor bearing host was still unknown. One hypothesis was that a mass of ROS in S180 tumor bearers was produced by tumor itself. Another hypothesis was that a specific factor that produced by tumor might suppress antioxidant productions such as SOD in S180 tumor bearers, leading to redundant ROS presence in blood and thymus, causing thymocyte apoptosis and thymic atrophy. Thus, the mechanisms of ROS-associated thymic involution in S180 tumor bearing mice needed to be further investigated.

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