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Immunomodulatory Effects of Aqueous Extract of Velvet Antler (*Cervus elaphus* Linnaeus) and Its Simulated Gastrointestinal Digests on Immune Cells *In Vitro*

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ABSTRACT

Velvet antler, a traditional Chinese medicine, is believed to have body-strengthening, immunomodulatory and anti-aging effects. It has been used in Chinese commercial functional foods and nutraceuticals. This study aimed to investigate the immunomodulatory effects of aqueous extract of velvet antler (AEVA) from *Cervus elaphus* Linnaeus and its simulated gastrointestinal digests (SGDs) *in vitro*. Experiments were carried out by measuring the effects of AEVA and SGDs on splenocyte proliferation, neutral red pinocytosis and nitric oxide (NO) production by macrophages. AEVA (10-400 μg/mL) showed inhibitory effects on concanavalin A (ConA)-stimulated splenocyte proliferation while no difference was observed when lipopolisaccharide (LPS) was used as stimulus. An inhibitory effect was also observed in neutral red pinocytosis by AEVA treated macrophages. During simulated gastrointestinal digestion, AEVA kept and strengthened its inhibitory effect on ConA-stimulated splenocyte proliferation. However, digestion of AEVA counteracted the inhibitory effect on pinocytosis. In addition, AEVA alone had no effect on NO production by macrophages while some SGDs (200 μg/mL) increased NO production by LPS-stimulated macrophages. The results indicated that AEVA could keep its inhibitory effect on T-cells by oral administration. In contrast, the effect of AEVA on macrophage function was changed during digestion.

Key words: *Cervus elaphus* Linnaeus, velvet antler, simulated gastrointestinal digestion, splenocyte proliferation, macrophages, pinocytosis, nitric oxide.

INTRODUCTION

Velvet antler cut from male cervids is commonly known as "Lurong" in China. Velvet antler is the growing stage of the horns borne on the heads of male members of the deer family. It grows at a very fast rate of up to 2 cm/day in some species and is a renewable resource. During the optimum regeneration period, velvet antler is removed and processed with no harm to the animal. Velvet antler has been used as a preventative agent to treat a wide range of ailments in traditional Chinese medicine (TCM) for more than 2000 years. It is believed to have many health benefits such as energy and growth enhancement[1], immunomodulatory function, anti-inflammatory properties[2], haemotopoietic effect[3], anti-aging effect[4], chronic wound healing effect, blood pressure modulation and cholesterol reduction[5].

Velvet antler has been used in many domains including medicine, nutraceuticals and functional foods. Previous studies focused on the ethanol-soluble extract of velvet antler. However, most components in velvet antler are water-soluble. The main components identified in aqueous extract of velvet antler (AEVA) include proteins, polypeptides, free amino acids, glycosaminoglycans (GAGs) and uronic acid[6]. Previous reports have shown the effectiveness of AEVA in treating various diseases[7,8,9], while other studies have focused on the immunomodulatory actions of AEVA. Recently, AEVA has been used in the treatment of some immune-related diseases (e.g. rheumatoid arthritis). In a previous study, AEVA was observed to inhibit T-cell activation strongly including blastogenesis and cytokine production in response to antigenic stimulation *in vitro*[9]. Intraperitoneal injection of AEVA into rats suppressed clonal expansion of helper T-cells[10]. AEVA was also observed to suppress the development of arthritis, even when it was administered after a booster immunization of colla-

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gen. In addition, it was proven to be a novel immunosuppressant which inhibited dihydroorotate dehydrogenase (EC 1.3.99.11)[11]. There was evidence that AEVA could reduce the chronic inflammation in adjuvant-induced arthritic rats[12]. Moreover, AEVA inhibited production of interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) from macrophages in response to *in vivo* stimulation with lipopolysaccharide (LPS) when it was administered into mice[9]. Although AEVA is believed to have an immunosuppressive effect mainly by intraperitoneal injection, it is unclear that whether AEVA could maintain its immunosuppressive activity when people conventionally take it orally during a gastrointestinal digestion process. Furthermore, systematic study on the immunomodulating activity of AEVA by immune cells *in vitro* is also lacking.

In order to further investigate the presence of immunomodulatory substances in AEVA and determine the immunomodulating activities before and after gastrointestinal digestion, AEVA was subjected to a gastrointestinal enzymatic hydrolysis process which simulated physiological digestion. The present study focused on the effects of AEVA and its simulated gastrointestinal digests (SGDs) on the *in vitro* proliferation of resting, Concanavalin A (ConA) and LPS-stimulated murine splenocytes as well as on the neutral red pinocytosis and nitric oxide (NO) production of peritoneal macrophages.

MATERIALS AND METHODS

I. *Materials*

Velvet antlers (*Cervus elaphus* Linnaeus) were obtained from male red deer that were bred at the Daxing'an Mountain range (Heilongjiang, China). Samples were freeze-dried, homogenized, and stored at 4°C. Pepsin (EC 3.4.23.1), pancreatin, ConA, LPS were from Sigma (St. Louis, MO, USA). RPMI-1640 medium and fetal bovine serum (FBS) was from Gibco BRL (Grand Island, NY, USA). All other chemicals were of analytical grade unless otherwise mentioned.

II. *Extraction and Preparation of AEVA*

Submicron powder of velvet antler (10 g) was extracted three times with 200 mL of distilled water in boiling-water bath for 2 h and then filtrated. The filtrate was concentrated at reduced pressure for convenience. The aqueous extract was freeze-dried and stored at 4°C for further use. The yield of AEVA was 40.2% (w/w of the dried sample).

III. *Simulation of Gastrointestinal Digestion*[13,14]

The freeze-dried AEVA was solubilized in water (5%, w/v). The solution was hydrolyzed at 37°C with pepsin using an enzyme: substrate ratio of 1:100 (w/w) and acidified with 10 M HCl to pH 2.0. The mixtures were then held in a thermostatic water bath for 4 h at 37°C under constant stirring.

After pepsin digestion, the pH of the mixture was raised to 6.8 with 10 M NaOH. Then pancreatin (20 mg/g AEVA) was added and the mixture was shaken for 1 min and left in a thermostatic water bath with pancreatin for 8 h at 37°C under constant stirring. During the enzymatic hydrolysis reaction, a pH-stat technique was used to maintain the mixture at a constant pH value using 1 M HCl or 1 M NaOH[15].

After aliquot was withdrawn after hydrolysis with pepsin, the pH was raised to 7.0 with 1 M NaOH. During hydrolysis with pancreatin, aliquots were withdrawn once per hour. The enzyme in each sample was inactivated by heating in boiling water for 10 min, followed by cooling to room temperature; the suspension was transferred to polypropylene tube and centrifuged at $10,000 \times g$ for 20 min. All supernatants were freeze-dried and stored at 4°C for further use.

IV. *Degree of Hydrolysis*

Degree of hydrolysis (DH), based on the reaction of α-amino groups, was calculated by reaction with ninhydrin according to the procedure published by Panasiuk *et al.*[16] with some modifications. Each tube contained 2 mL of test sample and 1 mL of freshly prepared ninhydrin solution (0.5% ninhydrin, 11.2% $Na_2HPO_4 \cdot 12H_2O$, 6% KH_2PO_4 and 0.3% fructose)[17]. Then the tubes were heated in a boiling water bath for 15 min. After cooling, the samples were mixed with 5 mL of 40% (v/v) ethanol and stayed for another 15 min. The absorbance was measured at 570 nm. The standard curve was prepared with L-leucine.

The degree of hydrolysis was calculated as:

$$DH\,(\%) = \frac{h}{h_{tot}} \times 100$$

$$= \frac{\alpha\text{-}NH_2 \text{ in SGDs} - \alpha\text{-}NH_2 \text{ in AEVA}}{h_{tot}} \times 100 \qquad [1]$$

h: Number of peptide bonds cleaved (m mol/g),
h_{tot}: Total number of peptide bonds (m mol/g).

V. *Animals*

Inbred male BALB/c mice (specific pathogen free, SPF), 6-8 weeks of age, were purchased from the laboratory animal center of the Academy of Military Medical Sciences and were housed under standard conditions (23 ± 2°C, 50%-60% relative humidity and 12 h light/dark photoperiod). They were provided with a rodent pellet diet and water *ad libitum*. All mice were allowed to acclimatize in the facility for 1 week before experiment. All experiments were carried out according to the National

Institutes of Health Guide for Care and Use of Laboratory Animals.

VI. *Preparation of Splenocyte Suspensions*

After sacrificed by cervical decapitation, mouse was soaked with 75% ethanol to reduce the chance of surface contamination. The spleen was aseptically removed and minced with a set of fine-tipped scissors and then pushed through a sterile wire mesh (200-mesh) in order to disperse splenocytes from tissue, and the resulting cells were suspended in cold phosphate-buffered saline (PBS). The cells were centrifuged ($257 \times g$) at room temperature for 5 min. The red blood cells were lysed by treatment with 3 mL of red blood cell lysis buffer (Beyotime, China) for 2 min. After washing twice with PBS, Erythrocytes-free splenocytes were then resuspended in RPMI-1640 medium, which was supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin and 10 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES). The splenocyte counts in a single cell suspension were determined with a hemocytometer and the suspension was adjusted to a working concentration of 2×10^6 cells/mL. Cell viability of greater than 95% was recorded after trypan blue staining.

VII. *Assay of Splenocyte Proliferation*

One hundred microliters of cell suspensions (2×10^6 cells/mL) were added in sextuplicate into the wells of a 96-well flat bottomed microplate (Costar) in medium with or without a suboptimal concentration of the T-cell mitogen ConA (5 μg/mL) or the B-cell mitogen LPS (5 μg/mL), together with AEVA or SGDs at various concentration (1, 10, 50, 100, 200 and 400 μg/mL) for a total of 72 h in a humidified atmosphere at 37°C in a 5% CO_2 incubator. Cell proliferation was assayed using Cell Counting Kit-8 (WST-8, Beyotime, China) according to the manufacturer's protocol. Incubation was continued for another 4 h. The absorbance was measured at 450 nm on a 96-well plate reader (ELISA reader, Thermo Elactron Inc., USA). Data are expressed as a stimulation index (SI) and were calculated as follows:

$$SI\,(\text{without ConA}) = \frac{OD_{cells+sample} - OD_{medium}}{OD_{cells} - OD_{medium}} \qquad [2]$$

$$SI\,(\text{with LPS}) = \frac{OD_{cells+sample+LPS} - OD_{medium+LPS}}{OD_{cells} - OD_{medium}} \qquad [3]$$

$$SI\,(\text{with ConA}) = \frac{OD_{cells+sample+ConA} - OD_{medium+ConA}}{OD_{cells} - OD_{medium}} \qquad [4]$$

VIII. *Preparation of Murine Peritoneal Macrophages*

Peritoneal macrophages were obtained from mice that had been injected intraperitoneally 3 days previously with 1.5 mL of 4% starch broth solution. The mice were sacrificed by cervical decapitation and soaked with 75% ethanol. A transverse cut was made in the inguinal area and the skin was dissected to expose the abdominal wall which was then swabbed thoroughly wet with 75% ethanol. Approximately 4 mL of cold RPMI-1640 medium was injected into the peritoneum of BALB/c mice. The needle was removed and the abdomen was gently massaged. Contents of the peritonea were transferred to ice-cold tubes. This procedure was repeated two more times. The cells were centrifuged at $257 \times g$ for 8 min and then adjusted to a concentration of 1×10^6 cells/mL with RPMI-1640 medium, buffered with 10 mM HEPES, supplemented with 10% heat-inactived FBS. Cells (100 μL) were seeded per well at a concentration of 1×10^6 cells/mL in 96-well flat bottomed microplate. After 2.5 h of incubation at 37°C in an atmosphere of 5% CO_2, the non-adherent cells were removed by vigorous washing (3 times) with warm serum-free medium and the adherent cells were further incubated in complete medium (RPMI-1640 medium supplemented with 10% heat-inactived FBS).

IX. *Assay of Pinocytosis by Peritoneal Macrophages*

The peritoneal macrophages in the absence or presence of stimulus (LPS at the final concentration of 30 μg/mL) together with different concentrations (1, 10, 50, 100, 200 and 400 μg/mL) of AEVA or SGDs were co-plated in 96-well plates. The plates were incubated at 37°C in a humidified incubator with 5% CO_2 for 24 h.

After incubation, the supernatant was collected and used to determine NO production. Then 200 μL of 0.1% neutral red (prepared in RPMI-1640 and filtered) was added in each well. Following 1 h of incubation at 37°C, the supernatant was discarded and each well was washed thrice with warm PBS and blot up with filter paper. Lysing solution (200 μL, 1% (v/v) acetic acid in 50% (v/v) ethanol) was added to each well and kept 2-3 h at room temperature. The absorbance of released dye was then measured at 550 nm using ELISA reader.

Data are expressed as a pinocytosis index (PI) and were calculated using the following equations.

$$PI\,(\text{without LPS}) = \frac{OD_{cells+sample}}{OD_{cells}} \qquad [5]$$

$$PI\,(\text{with LPS}) = \frac{OD_{cells+sample+LPS}}{OD_{cells+LPS}} \qquad [6]$$

X. *Assay of NO Production by Peritoneal Macrophages*

NO production was determined by measuring the accumulation of nitrite in the supernatant using Nitric Oxide Assay Kit (Beyotime, China) according to the manufacturer's protocol. Briefly, 50 μL of supernatant was added to 100 μL of the Griess reagent in 96-well plate. After incubation at room temperature for 10 min,

absorbance at 550 nm was determined in ELISA reader. Nitrite concentration was determined by using $NaNO_2$ as a standard.

XI. *Statistical Analysis*

Each set of experiments was carried out with six replicates. All experiments were repeated at least three times to check consistency of the results. Data were expressed as means ± SD. Statistical analysis was performed by student's t-test to express the difference between two groups. $P < 0.05$ was considered statistically significant. The correlation was calculated under Pearson correlation coefficient (2-tailed). Analysis was done with SPSS 13.0 (SPSS, Inc., Chicago, IL, USA).

RESULTS

I. *DH Changes During Simulated Gastrointestinal Digestion*

In humans, food stays in the stomach for about 4 h. After being processed by pepsin, food changes into chyme which goes on to the small intestine and stays 3-8 h for further digestion. The simulated gastrointestinal digestion of AEVA was monitored by DH using the ninhydrin assay for up to 12 h, as shown in Figure 1. In the peptic digestion process, only slight change in DH value was found. Upon further digestion by pancreatin, the initiative digestion rate increased significantly and slowed down thereafter. Stable DH values (~7%) were reached after 8 h, which indicated that maximum cleavage of peptide bounds occurred within 8 h of digestion. No significant differences were observed between the various SGDs in terms of the DH values during 8-11 h. It was indicated that
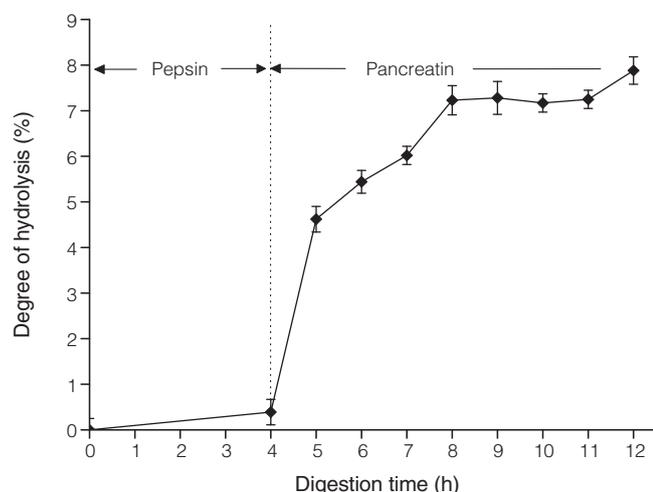
immunomodulatory peptides generated at the late period of digestion kept stable against pancreatin.

II. *Effect of AEVA on Splenocyte Proliferation In Vitro*

The effects of AEVA and SGDs on murine splenocyte proliferation, expressed as SI, were evaluated in the absence and presence of a suboptimal concentration (5 µg/mL) of ConA or LPS (Figure 2). AEVA stimulated the proliferation of resting splenocytes in a dose-dependent manner (r = 0.936, $P < 0.01$) up to the highest concentration used (400 µg/mL). Compared with cells as control, the stimulating effect became significant ($P < 0.05$) at 10 µg/mL, showing a SI value of 1.100. At concentration of 400 µg/mL, the SI value for AEVA was 1.328.

In order to study the immunomodulatory activity of AEVA, the ConA- and LPS-stimulated proliferations of splenolytes were measured. The ConA response was significantly suppressed (Figure 2) in a dose-dependent manner (1-400 µg/mL) showing that a significant correlation coefficient of 0.967 ($P < 0.01$). AEVA (10-400 µg/mL) stimulated the proliferation of resting murine splenocytes, but significantly inhibited the proliferation with 5 µg/mL of ConA. The inhibitory effect of AEVA was not due to cytotoxicity since the viability of cells treated with AEVA was higher than the control. The SI values for the control (Cells + ConA) and AEVA (400 µg/mL) were 2.565 and 1.850, respectively.

Despite AEVA inhibited the proliferation of splenocytes in the presence of 5 µg/mL ConA, it had no such effect in the presence of 5 µg/mL LPS (Figure 2).

III. *Effects of SGDs on Splenocyte Proliferation In Vitro*

Proliferations of splenocytes from inbred BALB/c

Figure 1. DH changes during simulated gastrointestinal digestion of AEVA.
Data are presented as means ± SD (n = 3).

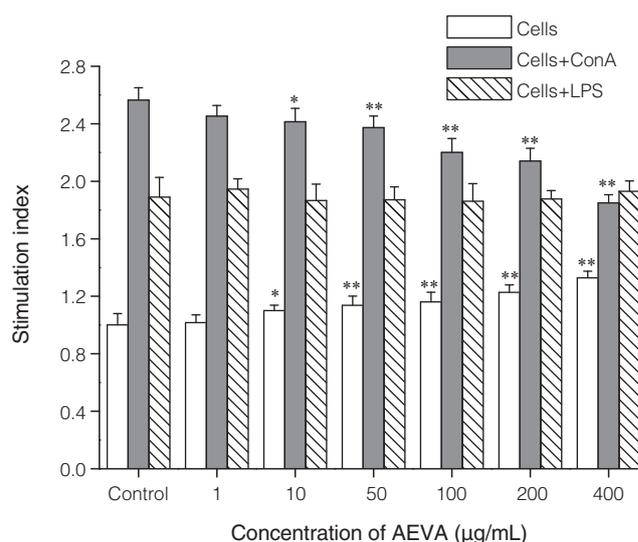**Figure 2.** Effect of AEVA on the proliferation of murine splenocytes. Data are presented as means ± SD (n = 6). *$P < 0.05$, **$P < 0.01$ (compared to the control group).

mice cultured under different SGDs were determined. All SGDs were evaluated in non-stimulated cells (without mitogen). Following simulated gastrointestinal digestion, AEVA lost its stimulating effect on splenocyte proliferation. SGDs (1-50 µg/mL) *in vitro* did not have any effects on the proliferation of resting splenocytes (data not shown).

The effects of AEVA and SGDs on ConA-stimulated murine splenocyte proliferation are shown in Figure 3. AEVA at 1 µg/mL did not significantly affect splenocyte proliferation, but SGDs obtained in 9-12 h significantly inhibited splenocyte proliferation (Figure 3A). At concentrations of 10 and 50 µg/mL, both AEVA and SGDs demonstrated significantly inhibitory effects on splenocyte proliferation in response to ConA (Figure 3B, 3C). Furthermore, the inhibitory effect was strengthened during digestion. When the digestion time reached up to 9 h (DH values reached ~7%), the SI values reached a comparative low level and remained stable. The inhibi-

tory effects of SGDs obtained in 9-12 h were significantly stronger than AEVA, especially at 50 µg/mL. In present study, the SI of SGDs was significantly and negatively correlated with the DH at 10 µg/mL (r = -0.758, $P < 0.05$) and 50 µg/mL (r = -0.720, $P < 0.05$).

The proliferation responses against ConA stimulation were significantly suppressed by adding SGDs to the cultures. However, the LPS responses hardly changed. It was found that priming with SGDs *in vitro* caused no significant effects ($P > 0.05$) on LPS-stimulated murine splenocyte proliferation (data not shown).

IV. *Effect of AEVA on the Functions of Peritoneal Macrophages*

(I) *Neutral Red Pinocytosis*

To convert a resident macrophage to a "primed" state *in vivo*, macrophages are elicited by injection of starch broth solution. Different concentrations of AEVA were incubated with murine peritoneal macrophages. As shown in Figure 4, the PI value for AEVA significantly decreased at 400 µg/mL ($P < 0.05$). However, there were no significant effects at lower concentrations (1-200 µg/mL) of AEVA ($P > 0.05$).

Primed macrophages become fully activated in response to secondary stimuli (e.g. LPS). Activated macrophages are those possessing tumoricidal functions. Figure 4 showed the effect of AEVA on pinocytosis under LPS-stimulated experimental conditions. LPS-stimulated macrophages incubated with AEVA (1-400 µg/mL) showed a dose-dependent inhibitory activity (r = -0.978, $P < 0.01$) against the pinocytosis by macrophages. The inhibitory effect of AEVA became significant ($P < 0.05$)
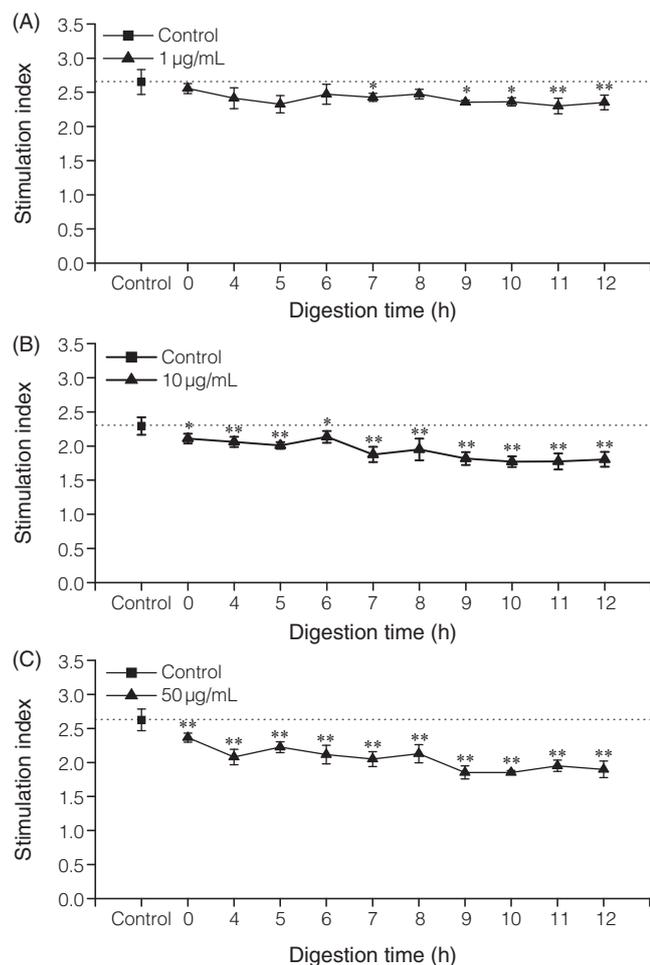

Figure 3. Effect of AEVA on the ConA-stimulated proliferation of murine splenocytes during simulated gastrointestinal digestion. Sequential digestion with two proteases was carried out for 12 h. Digestion was first carried out with pepsin for 4 h, and then for another 8 h with pancreatin. Data are presented as means ± SD (n=6). *$P < 0.05$, **$P < 0.01$ (compared to the control group).

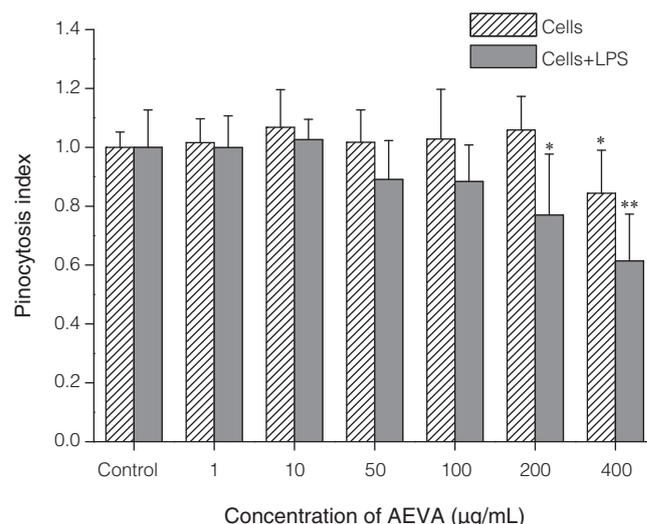**Figure 4.** Effect of AEVA on neutral red pinocytosis by murine peritoneal macrophages. Data are presented as means ± SD (n = 6). *$P < 0.05$, **$P < 0.01$ (compared to the control group).

at 200 μg/mL, showing a SI value of 0.770. At concentration of 400 μg/mL, the PI value for AEVA after 24 h incubation was 0.614.

(II) *Production of NO*

LPS, a component of the gram negative cell wall, was used to stimulate peritoneal macrophages[18]. Once activated, macrophages could produce a large number of cytotoxic molecules including NO, which is believed to be essential to take an important place in the microbicidal functions of macrophages. Since NO is a very labile molecule, its direct measurement in the biological samples is very difficult. In an aqueous solution, NO reacts with molecular oxygen and accumulates as nitrite and nitrate ions. Therefore, NO production was determined by measuring the accumulation of nitrite in the supernatant. Our results showed that NO production in the AEVA treated group was similar to that in the control group (Figure 5).

V. *Effects of SGDs on the Functions of Peritoneal Macrophages*

(I) *Neutral Red Pinocytosis*

The effects of SGDs on pinocytosis by primed macrophages are shown in Table 1. Neither AEVA nor SGDs at 100 μg/mL had significant effects on PI value. However, SGDs at 200 μg/mL was found to enhance the pinocytic activity, and the PI values of SGDs were signif-

icantly higher than the control. At 400 μg/mL, AEVA significantly inhibited pinocytosis by macrophages ($P < 0.05$, PI = 0.87), but SGDs did not. Compared with AEVA, the PI values were significantly elevated in several SGDs at 400 μg/mL.

The PI values of SGDs in LPS-stimulated macrophage models were also determined (Table 2). At concentrations of 100 and 200 μg/mL, the PI values of
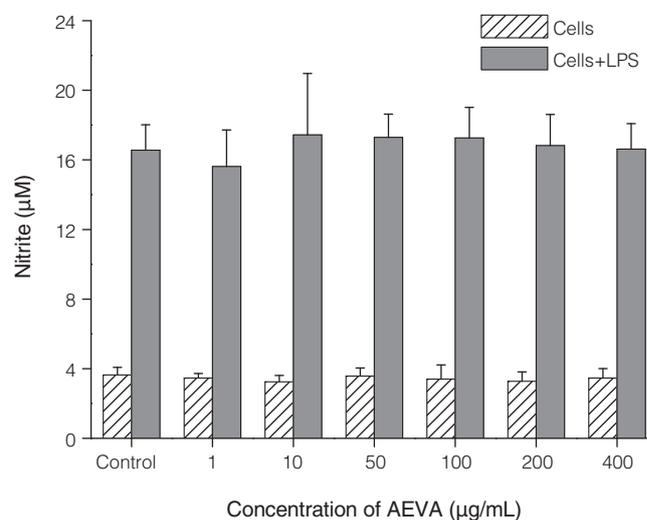

Figure 5. Effect of AEVA on NO production by murine peritoneal macrophages.
Data are presented as means ± SD (n = 6). *$P < 0.05$, **$P < 0.01$ (compared to the control group).

Table 1. Effects of AEVA and SGDs on neutral red pinocytosis by murine peritoneal macrophages *in vitro*

Sample	Digestion Time (h)	Pinocytosis Index		
		100 (μg/mL)	200 (μg/mL)	400 (μg/mL)
Control	—	1.00 ± 0.06	1.00 ± 0.10	1.00 ± 0.09
AEVA	0	1.01 ± 0.15	1.04 ± 0.19	0.87 ± 0.11*
	4	1.07 ± 0.17	1.07 ± 0.17	1.02 ± 0.13 #
	5	1.07 ± 0.08	1.22 ± 0.22*	1.18 ± 0.20 ##
	6	1.01 ± 0.11	1.07 ± 0.20	1.00 ± 0.09 #
	7	1.00 ± 0.11	1.19 ± 0.16*	0.92 ± 0.07
SGDs	8	1.07 ± 0.10	1.30 ± 0.23*	0.95 ± 0.15
	9	1.08 ± 0.19	1.27 ± 0.22*	1.04 ± 0.15 #
	10	1.09 ± 0.07*	1.40 ± 0.15**, ##	1.04 ± 0.11 #
	11	1.03 ± 0.16	1.23 ± 0.21*	0.87 ± 0.16
	12	0.98 ± 0.10	1.06 ± 0.22	0.72 ± 0.14*

Data are presented as means ± SD (n = 6).
Control group was untreated.
*$P < 0.05$, **$P < 0.01$ (compared to the control group).
#$P < 0.05$, ##$P < 0.01$ (compared to AEVA).

SGDs were similar to the control. However, both AEVA and SGDs at 400 μg/mL significantly inhibited pinocytosis in the presence of LPS ($P < 0.01$). The PI values for AEVA and SGDs at 400 μg/mL ranged between 0.58-0.74. Compared with AEVA, there were significant increases in PI at 200 μg/mL of SGDs, but no significant changes were observed at 400 μg/mL. The inhibitory effect of AEVA at 200 μg/mL on pinocytosis by LPS-stimulated macrophages was lost during digestion.

(II) *Production of NO*

Neither AEVA nor SGDs alone could initiate the production of NO by macrophages (Table 3). Some SGDs even slightly reduced NO production compared with the control.

The effects of SGDs on NO production induced by LPS at different concentrations are shown in Table 4. At concentrations of 100 and 400 μg/mL, NO production in the SGDs treated groups were similar to the control. However, NO production induced by LPS was enhanced by SGDs at 200 μg/mL. Most SGDs showed significant stimulating effects on NO production compared with the control and AEVA. In addition, SGDs obtained in 8-11 h had similar effects on NO production. The trend of NO production was consistent with that of neutral red pinocytosis test.

DISCUSSION

There has been a rapid development in the use of

TCM in recent years around the globe. Velvet antler is considered the second most important ingredient in TCM after ginseng. Some studies have focused on the immunomodulatory actions of AEVA. Intraperitoneal injection of AEVA was observed to inhibit T-cells and macrophages activation in recent years[9,10]. However, oral consumption of AEVA will lead to a gastrointestinal digestion process which could break down macromolecular substances, especially proteins. To the best of our knowledge, few investigations have addressed the immunomodulatory effects of AEVA during digestion. In the present study, SGDs were prepared by digesting AEVA using simulated gastrointestinal digestion method for different periods of time. The immunomodulatory effects of AEVA and SGDs obtained from velvet antler of *Cervus elaphus* Linnaeus on immune cells were investigated *in vitro*.

Splenocyte stimulation with mitogens is a measure of cell-mediated immunity. Meanwhile, ConA is a well known activator of T-cells, which induces the interleukin-2 (IL-2)/IL-2 receptor complex[19]. As shown in the results section, AEVA (10-400 μg/mL) significantly inhibited ConA-stimulated murine splenocyte proliferation with a significant dose-effect relationship (r = -0.967, $P < 0.01$). The results suggested that AEVA effectively suppressed T-cell function. During the 12 h-period of simulated gastrointestinal digestion, AEVA kept its inhibitory effect on ConA-stimulated splenocyte proliferation. The inhibitory effects of SGDs became much stronger than AEVA, especially at the late period of digestion (9-12 h). The likely presumptions of the inhibitory effects of AEVA and SGDs on T-cells are as follows:

Table 2. Effects of AEVA and SGDs on neutral red pinocytosis by LPS-stimulated murine peritoneal macrophages *in vitro*

Sample	Digestion Time (h)	Pinocytosis Index		
		100 (μg/mL)	200 (μg/mL)	400 (μg/mL)
Control	—	1.00 ± 0.22	1.00 ± 0.12	1.00 ± 0.16
AEVA	0	0.85 ± 0.16	0.71 ± 0.14**	0.65 ± 0.12**
	4	0.93 ± 0.18	0.88 ± 0.14	0.67 ± 0.09**
	5	0.98 ± 0.11	1.13 ± 0.16 ##	0.58 ± 0.10**
	6	1.12 ± 0.22 #	1.14 ± 0.14 ##	0.69 ± 0.13**
	7	0.99 ± 0.20	0.97 ± 0.16 #	0.65 ± 0.11**
SGDs	8	0.90 ± 0.12	1.01 ± 0.14 ##	0.73 ± 0.12**
	9	0.84 ± 0.10	1.00 ± 0.15 ##	0.66 ± 0.10**
	10	1.00 ± 0.17	1.15 ± 0.14 ##	0.74 ± 0.11**
	11	0.94 ± 0.15	1.00 ± 0.15 ##	0.65 ± 0.12**
	12	0.98 ± 0.19	1.02 ± 0.14 ##	0.60 ± 0.10**

Data are presented as means ± SD (n = 6).
Control group was treated with LPS (30 μg/mL).
*$P < 0.05$, **$P < 0.01$ (compared to the control group).
#$P < 0.05$, ##$P < 0.01$ (compared to AEVA).
#$P < 0.05$, ##$P < 0.01$

Table 3. Effects of AEVA and SGDs on NO production by murine peritoneal macrophages *in vitro*

Sample	Digestion Time (h)	NO Production (nitrite μM)		
		100 (μg/mL)	200 (μg/mL)	400 (μg/mL)
Control	—	4.37 ± 0.69	4.94 ± 0.26	4.12 ± 0.65
AEVA	0	4.11 ± 0.78	4.76 ± 0.30	3.90 ± 0.46
	4	4.06 ± 0.78	4.33 ± 0.33*	3.60 ± 0.62
	5	4.28 ± 0.46	4.54 ± 0.69	4.25 ± 0.60
	6	3.40 ± 0.10*	3.93 ± 0.51*, #	3.55 ± 0.39
	7	3.80 ± 0.39	4.41 ± 0.75	3.82 ± 0.58
SGDs	8	3.18 ± 0.39*	4.24 ± 0.50*	4.17 ± 0.60
	9	4.06 ± 0.71	4.63 ± 0.84	4.17 ± 0.26
	10	4.02 ± 0.52	4.59 ± 0.82	4.30 ± 0.65
	11	3.80 ± 0.56	4.59 ± 0.39	4.65 ± 0.55
	12	4.02 ± 0.71	4.68 ± 0.68	4.60 ± 0.51

Data are presented as means ± SD (n = 6).
Control group was untreated.
*$P < 0.05$, **$P < 0.01$ (compared to the control group).
#$P < 0.05$, ##$P < 0.01$ (compared to AEVA).

Table 4. Effects of AEVA and SGDs on NO production by LPS-stimulated murine peritoneal macrophages *in vitro*

Sample	Digestion Time (h)	NO Production (nitrite μM)		
		100 (μg/mL)	200 (μg/mL)	400 (μg/mL)
Control	—	17.18 ± 1.26	18.31 ± 2.36	18.55 ± 1.76
AEVA	0	16.17 ± 1.63	18.80 ± 0.72	18.70 ± 1.88
	4	16.69 ± 1.09	21.51 ± 1.19 ##	21.44 ± 2.14*, #
	5	17.04 ± 1.28	23.76 ± 2.23*, ##	20.71 ± 2.35
	6	18.51 ± 2.58	21.88 ± 3.64	19.84 ± 1.33
	7	15.60 ± 1.48	23.60 ± 4.09	20.54 ± 1.96
SGDs	8	16.39 ± 2.06	22.41 ± 0.82*, ##	19.46 ± 2.72
	9	15.33 ± 1.16*	23.84 ± 1.64**, ##	19.87 ± 0.96
	10	16.25 ± 2.16	23.48 ± 1.76*, ##	19.90 ± 1.97
	11	15.29 ± 0.84*	21.88 ± 1.07*, ##	19.14 ± 2.08
	12	15.68 ± 1.36	21.10 ± 2.33	19.69 ± 1.20

Data are presented as means ± SD (n = 6).
Control group was treated with LPS (30 μg/mL).
*$P < 0.05$, **$P < 0.01$ (compared to the control group).
#$P < 0.05$, ##$P < 0.01$ (compared to AEVA).

(1) both originally carry immunosuppressive compo-nents; (2) peptides were released during digestion; (3) interactions occured among effective components. Since AEVA kept its inhibitory effect before and after diges-tion, it was speculated that the original components in AEVA were more responsible for the inhibitory effects, and the components could keep their inhibitory effects after digestion. Proteins, the major components in AEVA, broke down into peptides and amino acids during gastro-intestinal digestion. Thus, bioactive peptides released

during digestion might contribute to the enhancement of inhibitory effects. Recently, the response by T-cells is considered not only a factor in acute infections but also an integral component of biological processes such as development and aging as well as the pathophysiology of many chronic diseases (e.g. rheumatoid arthritis, type I diabetes, celiac disease, cancer, and cardiovascular disease)[20-22]. Therefore, AEVA might be useful for the treatment of these diseases by oral administration as well as injection.

In addition, B-cells are also critically important in the severity and length of the diseases[23]. AEVA and SGDs had no effects on the LPS response of murine splenocytes since LPS is an activator of T-cell-dependent antibody production by B-cells. Contact and signaling between B- and T-cells can then result in B-cell proliferation[24]. It was speculated that AEVA and SGDs could down-regulated the proper cytokines expression derived from T-cells or decreased the contact between B- and T-cells.

Macrophages act as pivotal immunocytes of the host defense against invading microbes and tumor growth. The potential immunomodulating activities of AEVA and SGDs to both primed and activated murine peritoneal macrophages, as determined by neutral red pinocytosis and NO production were also investigated. The results demonstrated that AEVA treated macrophages showed reduced pinocytosis at higher concentrations. This result was consistent with the report by Kim *et al.*[9]. However, an elevated effect on pinocytosis by macrophages was observed during simulated gastrointestinal digestion of AEVA. The inhibitory effect might result from the original components in AEVA, whereas the increases in pinocytic activities of SGDs could be due to immunomodulatory peptides and amino acids released during digestion.

Bi-directional regulation is a biphasic dose-response relationship characterized by low dose stimulation, high dose inhibition, resulting in either a J-shaped or an inverted U-shaped dose response. Some natural extracts and chemicals (either natural or synthetic) have been found to show bi-directional regulatory effects[25-27], often occurring in TCM. The pharmacological effects of TCM depend on the combined action of its complex compositions. TCM shows dose-effect relationship when its chemical compositions have consistent pharmacological effects. However, if the chemical compositions have reverse action, the final effects will rely on the dose ratio and the respective increasing rate of pharmacological effects of these two compounds. The effects of SGDs on pinocytosis by macrophages could be attributed to the interactions between the originally immunosuppressive components in AEVA and immunomodulatory peptides released during digestion. According to the results of neutral red pinocytosis by primed macrophages (Table 1), SGDs at 200 μg/mL showed stimulating effects on pinocytosis. It was supposed that the originally immunosuppressive components at lower concentrations could not affect pinocytic activity. Thus, immunomodulatory

peptides released during digestion could account for the stimulating effects. When the concentration increased to 400 μg/mL, SGDs showed no significant pinocytic activities during the whole digestion. It was speculated that immunomodulatory peptides released during digestion could counteract the inhibitory effect of the originally immunosuppressive components. Furthermore, the counteractive effect by immunomodulatory peptides could also explain the pinocytic activities in LPS-stimulated macrophage models with SGDs, especially at 200 μg/mL (Table 2). However, at 400 μg/mL, the inhibitory effect of the originally immunosuppressive components was so strong that immunomodulatory peptides could not counteract it any more. Therefore, both AEVA and SGDs at 400 μg/mL showed inhibitory effects on pinocytosis in the presence of LPS.

NO has been shown to be the principal effector molecule produced by macrophages for cytotoxic activity and can be used as a quantitative index of macrophage activation[28]. AEVA administration in the two experimental models resulted in an uniform phenomenon. No significant effect on the production of NO was found. AEVA and SGDs alone could not elicit NO production, since NO is synthesized as needed by inducible nitric oxide synthase (iNOS) from its precursor L-arginine[29]. Under normal circumstances, macrophages possess no detectable iNOS (EC 1.14.13.39). Stimuli such as interferon-γ and LPS elicit new iNOS synthesis, mediating the NO responses to inflammatory stimuli[29]. The regulation of NO production leads to three possibilites: influence of iNOS expression and activity, influence of substrates availability and scavenging of generated NO. Since SGDs could not increase NO production in resting macrophages (Table 3), we speculated that SGDs alone could not induce the expression of iNOS. As we know, NO production by macrophages is bi-directionally regulated by various factors (lipophosphoglycan, 5,6-Dimethylxanthenone-4-acetic acid, swainsonine, picolinic acid, prolactin and growth hormone)[30-34]. In our study, AEVA had no significant effect on NO production induced by LPS (Figure 5). However, NO production by LPS-stimulated macrophages was significantly enhanced by SGDs at 200 μg/mL. There were no significant effects at low-dose (100 μg/mL) and at high-dose (400 μg/mL) of SGDs (Table 4). Thus, NO production in LPS-stimulated macrophage model was also bi-directionally regulated by SGDs. The effects of SGDs on NO production could be due to the bi-directional regulation of immunomodulatory peptides and amino acids released during digestion.

CONCLUSIONS

In conclusion, our present study has demonstrated that AEVA had potential immunosuppressive capabilities including the ability to inhibit ConA-stimulated splenocyte proliferation and the neutral red pinocytosis activ-

ity. During digestion, AEVA kept and strengthened its inhibitory effect on ConA-stimulated splenocyte proliferation. However, digestion of AEVA counteracted the inhibitory effect on pinocytosis, and SGDs at 200 µg/mL even showed stimulated effects in primed macrophages. Therefore, we speculated that some original components in AEVA were responsible for the immunosuppressive effects, and immunomodulatory peptides could be released during digestion. AEVA could keep its inhibitory effect on T-cells by oral administration. In contrast, the effect of AEVA on macrophage function was changed during digestion. To unravel potentially immunomodulatory actions of AEVA and SGDs, the bioactive components should be further clarified. Further investigation of the impact of bioactive components on the production of some cytokines in mitogen-stimulated lymphocytes and macrophages is also needed.

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