The Anti-stress Effects of Sarcandra glabra Extract on Restraint-Evoked Immunocompromise

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Sarcandra glabra was a renowned herb traditionally used as herbal tea or food supplement to enhance mental efficiency and to recover from stress or fatigue in China. We investigated the effects of Sarcandra glabra extract (SGE), with chemical composition clearly showed by HPLC fingerprint as quality control, on immunologic response including natural killer (NK) cell activity and its antioxidative capacity in splenocytes obtained from restrained mice. Our results found that daily oral administration of SGE (125, 500 mg/kg/d) for 5 consecutive days to restrained mice alleviated the stress-induced reduction of the number of lymphocytes, the balance of CD4+ T/CD8+ T and NK cell activity per spleen, SGE also significantly decreased the level of lipid peroxidation and increased oxygen radical absorbance capacity (ORAC) in splenocytes. These results indicated that SGE modulate stress-attenuated immunologic response, at least, partially explained by improving antioxidative capacity in immunocytes.

Key words Sarcandra glabra; restraint stress; natural killer cell activity; oxygen radical absorbance capacity; antioxidation

Stress and frustration can trigger problems in mental and physical health, which in turn can cause many life-style diseases over a prolonged period of time. Herbs, as a good source of vitamins, minerals, and antioxidants, have played an important role in stress management. Sarcandra glabra (Thunb.) Nakai (Chloranthaceae) was a renowned herb traditionally used as herbal tea or food supplement to remedy ailments, to enhance mental efficiency and to recover from stress or fatigue in China.1-3 It grows in the southern parts of China, Japan and southeastern Asia. The chemical compositions of Sarcandra glabra are volatile oil, phenolic acids, polysaccharide, etc.4-6 Isofraxidin, fumaric acid, terpenoid saponins and other flavonoids were isolated from the ethanolic extract of Sarcandra glabra.7 Its volatile oil is widely used in tooth-paste and chewing gum to inhibit oral bacteria.8 The whole plant is also used as herbal tea or supplementary food for its anti-infectious and anti-inflammatory effects, which are closely related to immunity.9 For these reasons, the research and development on Sarcandra glabra have attracted great attention in the international scientific community. Some studies have demonstrated that Sarcandra glabra exhibited immunological activities, which appeared to induce an increased protection against microbial and viral infections,6 cytoprotective activities,2 and anticancer activities.9 However, these data were obtained by experiments in vitro and little is known about whether Sarcandra glabra has any effects on animals.

To investigate the effects of Sarcandra glabra extract (SGE) on immunological response in vivo, we used restraint loaded mice as an animal model. Restraint is a common stress-causing factor that exerts great impacts on immunological system.8 It was reported that restraint stress in mice changed the number of lymphocyte cells, altered antibodies production, and suppressed cytotoxicity of natural killer (NK) and T cells.9-12 Fukui demonstrated that restraint stress-induced change in lymphocyte cell number closely correlated with the altered antibody and cytokine levels.11 Reports from Sheridan’s lab have shown that restraint stress suppressed T-cell cytokine production and cytolytic T cell activity in virus-infected animals.13 They also found that restraint stress significantly modulated NK cell trafficking and cytolytic activity and contributed to elevated virus replication.14 But the molecular mechanisms underlying the link between restraint stress and these immune dysfunctions remain elusive. It was reported that NK cells and T cells were susceptible to reactive oxygen species (ROS), and would lose their activities under oxidative stress.15,16 The critical parameters of oxidation are lipid peroxidation and its production of malondialdehyde (MDA).17 To estimate the ability of protection against oxygen free radicals, a sensitive and reliable method called oxygen radical absorbent capacity (ORAC) assay was used.18 Based on this knowledge, we detected the anti-stress effects of SGE on restraint-evoked immunocompromise in mice, by evaluating the effects of SGE on recovering the number of lymphocyte cells in spleen, the balance of T lymphocyte subpopulation (CD4+ T/CD8+ T) and NK cell activity in splenocytes of mice. We further hypothesized the mechanisms might be associated with its anti-oxidative effects in immune cells, by detecting the production of MDA and the level of ORAC of immune cells. On the other hand, we carried out HPLC fingerprint analysis to control the quality of SGE, and we also determined compound structures of the seven main chromatographic peaks in the fingerprint.

MATERIALS AND METHODS

Materials and Preparation of SGE 5-Caffeoylquinic acid, 3-caffeoylquinic acid, 4-caffeoylquinic acid, caffeic acid, isoferafidin, 4’-O-β-d-glycopyranosyl rosmarinic acid, and rosmarinic acid used in this study had previously been isolated from Sarcandra glabra. Mouse immunoglobulin (Ig) G1-fluorescein-isothiocyanate (FITC) or phycoerythrin (PE), anti-CD3 (FITC), anti-CD4 (PE), and anti-CD8 (PE) were all purchased from Beckman (U.S.A.). Phenylhydrazine and
thiobarbituric acid were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Sodium fluorescein (FL), 2',2'-azobis(2-amidinopropane)-dihydrochloride (AAPH), and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). *Sarcandra glabra* was generously provided by Guangzhou Jingxutang Pharmaceutical Co., Ltd. (Guangzhou, China), and authenticated by Professor Yao Xinsheng (College of Materia Medica, Jinan University). A voucher specimen (Z03107) was maintained in Institute of Traditional Chinese Medicine & Natural Products, Jinan University, Guangzhou 510632, China. The SGE was prepared as follows: 500 g crude dry plant was decocted with 5000 ml of boiling water for 40 min to half volume. The extracted solution was centrifuged at 3500 rpm for 30 min, and the supernatant was filtered and lyophilized.

**Reverse-Phase HPLC Analysis of SGE’s Chemical Composition** For the quality control of the oral administration to mice, SGE chemical pattern was obtained by the PDA HPLC analysis and indicated in Fig. 1. Compared with the standard compounds, seven main chromatographic peaks were identified as follows: 5-cafeoylquinic acid (1, *t* _R_ 9.0 min), 3-cafeoylquinic acid (2, *t* _R_ 14.9 min), 4-cafeoylquinic acid (3, *t* _R_ 16.6 min), caffeic acid (4, *t* _R_ 19.2 min), isoferixin (5, *t* _R_ 27.5 min), 4'-O-β-D-glycopyranosyl rosmarinic acid (6, *t* _R_ 35.3 min), rosmarinic acid (7, *t* _R_ 39.3 min).

**Animals and Treatment** Seven week-old male C57BL/6j mice purchased from the Center of Laboratory Animal Science Research of Southern Medical University (Guangdong, China), were kept in a specific pathogen-free animal room at 23 °C with a 12-h dark–light cycle and were fed with standard laboratory diet and tap water. The animals were allowed to acclimatize to the environment for 1 week before the experiment. The experimental procedure was shown as Fig. 2. Experimental groups received oral administration of SGE dissolved in drinking water at a final concentration of 12.5 and 50 mg/ml, while the normal control group and stress control group received water only. The intakes of SGE water solution were 0.1 ml per 10 g body weight for 5 d. On the second day of administration, mice were physically restrained in a 50 ml polypropylene centrifuge tube with holes for 12 h, and then placed in the home cage with food and water before the assay. After 3 d of recovery, all mice were sacrificed and the spleens were removed. The Institutional Laboratory Animal Care approved all animal procedures. All studies were conducted in accordance with the guidelines set forth by the National Institutes of Health and the U.S. Department of Agriculture.

**Tumor Cell Line** YAC-1 tumor cell line, a moloney virus induced mouse T cell lymphoma of A/SN origin, was obtained from Institute of Health Care Science (Suntory Ltd., Japan). The YAC-1 cell was used to test the NK cytotoxic activity for its noted sensitivity to NK cells. Cell line was cultured in RPMI-1640 containing 10% fetal bovine serum (FBS) at 37 °C in a humidified 5% CO₂ atmosphere before tested.

**Splenocyte Preparation** The spleens were collected and splenocytes were prepared by disrupting the spleen with a grinder in phosphate-buffered saline (PBS, pH 7.4). The total splenocyte number was determined with a blood–cell counter.
ity (one lytic unit, 1 LU10) was established from the best fit of the curve. The number of LU10 per spleen (LU10/spleen) was thus calculated and used to express final results. Assays for each effector/target cell ratio were performed in triplicate. After incubation for 4 h at 37 °C under a 5% CO2 atmosphere, lysis of target cells was calculated using Eq. 1.

\[ \text{lysis} (%) = \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100\% \]  

The maximum release referred to the lysis obtained after adding Triton X-100 (final concentration 1%). Spontaneous release was determined by the incubation of labeled target cells in the absence of effector cells.

**Thiobarbituric Acid-Reactive Substances (TBARS) Assay** The level of lipid peroxide in the splenocytes was determined by measuring TBARS. Splenocytes were homogenized in PBS at a concentration of about $2 \times 10^7$ cells/ml. The splenocyte lipid peroxide content was measured using a modified version of the method of Fraga. Briefly, 200 µl of 0.15 mM NaOH was added and 20 µl of this diluted splenocyte homogenate was incubated at 37 °C in a water bath with or without 10 µl of 0.3 mM phenylhydrazine (in 35% methanol). After 45 min incubation, 10 µl of 1% thiobarbituric acid in 1% acetic acid (pH 3.5) was added. The mixture was shaken vigorously and then heated at 95 °C for 45 min. After cooling, the samples were centrifuged at 3000 rpm for 10 min at 20 °C and the pink coloration of the supernatant was measured at 532 nm.

**ORAC Assay** The procedures for the ORAC assay on SGE *in vitro* and splenocytes were modified from the previously described method of Kurihara. This assay measures the effectiveness of antioxidant components in splenocytes and SGE, by inhibiting the decline of FL fluorescence induced by a peroxyl radical generator, AAPH. Automated ORAC assay was carried out on a Labsystems Fluoroskan Ascent plate reader (Helsinki, Finland) with fluorescent filters (Infinite™ F200, excitation wavelength, 485 nm; emission wavelength, 527 nm). Fluorescein was used as a target for free radical to attack and the reaction was initiated with AAPH, and Trolox was used as a control standard. Final results were calculated based on the difference in the area under the fluorescein decay curve between the blank and each sample.

**Statistical Evaluation** One-way analysis of variance (ANOVA) was applied to analyze the different groups, followed by Dunnett's post-hoc test for pair-wise multiple comparisons. Differences were considered as statistically significant at $p<0.05$.

**RESULTS**

**Effects of SGE on Splenocyte Counts and Lymphocyte Subsets in Restraint-Stressed Mice** As shown in Fig. 3A, restraint stress reduced the spleen cell number significantly ($p<0.01$), and oral administration of SGE (125, 500 mg/kg/d, 5 d) significantly improved the spleen cell number near to normal. In lymphocyte subpopulations, as shown in Fig. 3B, the ratios of CD4$^+$ T to CD8$^+$ T cells of the normal control...
group and stress control group were 2.28±0.26 and 1.62±0.14, and those of oral administration of SGE (125, 500 mg/kg/d, 5 d) were 1.80±0.19 and 1.95±0.3, respectively.

Effects of SGE on NK Cell Activity in Restraint-Stressed Mice The effects of SGE on the NK cytotoxic activity in spleen was assessed by a flow cytometer assay method using NK-cell-sensitive YAC-1 target cells (as illustrated in Fig. 4). We investigated the effects of SGE on the activity of NK cells 3 d after the stress loading, and results showed that the NK cell activity per spleen was suppressed (p<0.01). However, oral administration of SGE (125, 500 mg/kg/d, 5 d) improved the suppressed NK cell activity per spleen significantly (as shown in Fig. 5).

Inhibitory Effects of SGE on Splenocyte TBARS in Restraint-Stressed Mice The extent of lipid peroxidation in splenocytes was evaluated by the TBARS method. The basal value of lipid peroxide was 1.7±0.2 nm in splenocytes at a concentration of about 2×10^7 cells. It markedly increased to 2.9±0.2 nm/2×10^7 cells after restraint stress. Administration of SGE (125, 500 mg/kg/d) reduced the stress-induced increases in lipid peroxide to 2.4±0.1 and 2.1±0.3 nm/2×10^7 cells, respectively (Fig. 6).

Antioxidative Capacity of SGE in Vitro and in Vivo The ORAC value was calculated as the ratio of the area under the fluorescence decay curve for 500 μg/ml trolox as a standard. Figure 7 shows the working curves of fluorescein oxidation used as an index of resistance time for the oxidative reaction. The linear relationship between the net area and different concentrations of SGE was evaluated as shown in Fig. 7. In normal mice, the splenocytes ORAC level was
pressed function of NK cells, leading to an increased vulnerability to infections or to the occurrence of malignant tumors.\(^{12}\) These reports support our results, by suggesting that SGE markedly improved immunologic response was possibly due to its antioxidative capacity in immune cells.

According to traditional Chinese beliefs, Sarcandra glabra is a renowned herb as anti-infectious and anti-inflammatory supplement, which is closely related to immunologic response. Although no references described the mechanism, our results showed that SGE attenuates stress by increasing the number and activity of splenocytes in mice subjected to stress and improving antioxidative processes, through exerting antioxidative activity directly or indirectly. Because the major constituents of SGE are 5-cafeoylquinic acid, 3-cafeoylquinic acid, 4-cafeoylquinic acid, caffeic acid, isofraxidin, 4’-O-β-D-glycopyranosyl rosmarinic acid, rosmarinic acid, which are good antioxidants in vitro, and these constituents may mediate its biological effects in vivo.\(^{27–30}\) In present studies, the ORAC value of SGE in vitro also indicates that it is a potent antioxidant. Administration of SGE significantly inhibited the production of stress-induced lipid peroxide and augmented the ORAC activity. These results suggested that SGE improved stress-induced impairment of immunity via its antioxidative property which results in activated effects on anti-infection and anti-inflammation. Considered that the quality of SGE was controlled by HPLC fingerprint, and the previous acute toxicity and mutagenicity studies approved the dietary safety of SGE.\(^{31}\) Therefore we concluded that the water extract of Sarcandra glabra possesses a good efficacy and safety in anti-stress treatment.

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**REFERENCES**