Effects of H_2O_2, paraquat, and ethephon on herbal drug quality of *Schiandra chinensis* based on reactive oxygen species system

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ABSTRACT

Background: Nowadays, more and more herbal drugs of traditional Chinese medicine (TCM) rely on cultivation rather than natural resources because of overexploitation, and the study on quality of cultivated herbal medicines has become a hotspot in the research field of ecology of TCM resources. Though some of molecular biology techniques could improve the contents of secondary metabolites, those chemical compositions may differ from what we require from natural products, resulting in different treatment efficacy. **Objective:** To explore ways and means of improving TCM quality by means of regulating secondary metabolism from the perspective of natural physiological ecology. **Materials and Methods:** H_2O_2 and paraquat as carriers of ROS, propyl gallate as a ROS scavenger, and ethephon as a ROS inductive agent were sprayed on *Schisandra chinensis* (Turcz.) Baill. **Results:** The hypo-concentration ROS could enhance the activities of POD, PPO, as well as CAT, and propyl gallate acted on the opposite side, but they all failed to break the homeostasis between ROS and oxidase activity and to exert any effect on the contents of the schisandrin, deoxyschizandrin, and schisandrin B. The hypso-concentration ROS could break the homeostasis, reduce the activities of POD, PPO, as well as CAT, and improve the quality of *S. chinensis* fruit. The ethephon could effectively improve the quality of *S. chinensis* without the limitation of metabolic regulation. **Conclusion:** The conclusions accord with the hypothesis that ROS regulates secondary metabolism.

Key words: Ethephon, H_2O_2, metabolism, paraquat, propyl gallate, *Schisandra chinensis*

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INTRODUCTION

Secondary metabolism is a special metabolic process deriving from primary metabolite. Plant secondary metabolites, served as medicinal ingredients, have been used to treat various diseases for thousands of years in some countries, and about 25% of clinical drugs come from plant secondary metabolites directly or indirectly.

It has been proved that germplasm and environment are the major factors affecting the categories and contents of plant secondary metabolites.

Metabolic regulation to improve the contents of secondary products has become a very active research field, and is considered to be a new approach to solve low-yield constitutes in secondary products of plant cell cultivations. To date, some of molecular biological techniques are applied to increase the plant secondary metabolites, such as gene addition,[1] gene engineering of regulator genes and transcription factor,[2] antisensenucleic acids and RNA interference technique,[3] gene modification and so on. Secondary metabolites are determined by the synergism of various related enzyme activities, and affected by multiple steps such as signal transmission, transcription factor activities, synthetic genes expressions under the influence of inducer. Furthermore, from the point of view of current researches, efficacious effects of herbal medicine usually result from multicomponent synergism rather than one single constituent.[4] Therefore, it is difficult to evaluate the quality of material medica based on one or several chemical compounds,[5,6] and the contents of specified constituents induced by molecular biology techniques may not radically improve the quality of plant medicine on the purpose of

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improving efficacy due to differences in the proportion of ingredients from natural part.

Ecotope is another important factor to plant quality. Chinese people, as early as Tang dynasty of 1000 year ago, recognized that the quality of medicinal plant was susceptible to habitat environment, which gradually developed into a conception of famous-region drug, a synonym for excellent drug. The qualities of many herbal drugs are formed under ecological stress (adversity); however, most of the investigations nowadays just focus on the surface relationship between a concrete environmental factor and the quality of herbs, without lucubrating the relationship between metabolic pathways of plants and the environmental factors, which results in difficulty for quantization and intervention of environmental factors in practice. Therefore, the correlation between TCM quality, herbal metabolic pathways, and ecology environment has become a new spot to discuss the ecology research on the quality formation of natural resources of Chinese medicinal materials.[7] It is possible that the environment effecting on the unique qualities of famous-region drugs should be carried out through possible physiological processes based on some chemical compounds, and the studies of intrinsic mechanisms will be quite sufficient to remain cultivated quality in accordance with that in natural state.

Schisandra chinensis (Turcz.) Baill is widely distributed at mountains in the northeast China, Korea, Japan, and Russia. Its fruit, an important TCM in Asian nations such as China, Japan, and Korea, could stay caducity, tonic, sedative, and hepatoprotection. Now, its cultivated area is over 10 000 hectares in the northeast China. The drug quality is related with the place of origin, from which schizandrin varied from 0.41% to 0.81%, schisandrin B from 0.209% to 0.478%.[8,9] Therefore, the present study was designed to argue the role of ROS on secondary metabolism of *S. chinensis* and the strategies of excellent treatments in cultivation.

MATERIALS AND METHODS

Materials

The *S. chinensis* was identified by Dr. Xiangcai Meng, Heilongjiang University of Chinese Medicine, China. Samples of 5 years old *S. chinensis* were chosen from medicinal garden of Heilongjiang University of Chinese Medicine, planting density 0.5 m × 0.5 m, fence-style rack with height of 2.0 m. The *S. chinensis* were randomly divided into 10 groups (5.0 m per group, repeated three times). From July 10th to August 20th, H_2O_2 (10 mmol/l, 20 mmol/l) and propyl gallate (10 mmol/l, 1 mmol/l) were sprayed every day; paraquat (0.001%, 0.01%, and 0.1%) and ethephon (10 ppm, 1 ppm) were sprayed once every 3 days, and nothing was sprayed to the control group. A voucher specimen (W2010005) is deposited at the research center, Heilongjiang University of Chinese Medicine, China.

At well-lit area, 13-15 leaves of branch were picked to determine oxidase activities such as hydrogen peroxidase (CAT), peroxidase (POD), polyphenol oxidase (PPO) on August 10th; the fruit of *S. chinensis* were picked, dried in cool place indoors, to determine the contents of schisandrin, deoxyschizandrin, and schisandrin B on September 10th.

Methanol, HPLC grade, was purchased from Fisher Company, Inc. (New Jersey, USA). Distilled water was purchased from Qu Chenshi Company, Inc. (Guangzhou, China). The chemical compounds for schisandrin, deoxyschizandrin, and schisandrin B were obtained from the National Institute for the Control of Pharmaceutical and Biological Product (Beijing, China).

METHODS

Oxidase activity

All the leave samples in each group were divided into several parts according to usage amount for analysis, grinded below 0 °C to determine the activities of CAT, POD, and PPO. CAT activity was analyzed with ultraviolet spectrophotometry, POD with guaiacol method, and PPO with pyrogallic acid colorimetry, which are all conventional methods.

Active ingredients

Chromatography

Chromatographic separations were performed on a DiamonsilC18 column (4.6 nm × 200 mm, 5 μm) using an Acquity ultrahigh performance liquid chromatography system (Waters) equipped with a photodiode array detector (set at 250 nm). The column temperature was set at 40 °C and the gradient eluting program was started from 70% (methanol/H_2O) to 72% within 7 min, then from 72% to 76% within 2 min and held at 76% for 7 min. The total flow rate was 0.4 ml/min, injection volume 20 μl.

Sample preparation

Powder of *S. chinensis* fruit were accurately weighted, and put in 25 ml erlenmeyer flask, in which 10 ml methanol was added, sealed and extracted by ultrasonic extractor for 30 min, centrifuged at 3000 r/min for 15 min, and the supernatant liquid was filtrated through a 0.45 μm pore size filter.

Calibration curve

10.46 mg schizandrin, 9.50 mg deoxyschizandrin, and 10.65 mg schisandrin B are accurately weighted, placed

in 10 ml, 20 ml, 20 ml volumetric flasks, respectively, dissolved and diluted to the calibration tails with methanol. The calibration curves were constructed by plotting the schizandrin, deoxyschizandrin, and schisandrin B peak area against concentrations using the least-squares method.

Precision and accuracy

Draw 10 μl of above-mentioned sample solution to determine the contents of these compounds six times, peak area RSD for schizandrin, deoxyschizandrin, and schisandrin B was 0.32%, 2.06%, and 1.08%.

Stability

Draw 10 μl of the above-mentioned sample solution to determine the contents of these compounds once every 5 h for 5 times, peak area RSD for schizandrin, deoxyschizandrin, and schisandrin B was 0.45%, 1.86%, and 1.21%.

Repeatability

Five parallel samples were prepared, peak area RSD for schizandrin, deoxyschizandrin, and schisandrin B was 1.23%, 1.52%, and 1.38%, respectively.

Extract recovery

About 0.2 g drug sample are accurately weighted, placed a certain amount of schizandrin, deoxyschizandrin, and schisandrin B, extracted as the methods mentioned above. The average recoveries were 98.8%, 101.6%, and 100.7%, respectively.

RESULTS

For ROS carriers, H_2O_2 could increase the activities of CAT [Figure 1], decrease that of POD and PPO within 10–20 mmol/l range [Figures 2 and 3], and positively correlate with the contents of schizandrin, deoxyschizandrin, and schisandrin B [Figure 4a and b]; deoxyschizandrin in 20 mmol/l H_2O_2 group was significantly higher than that in control group, and schizandrin as the highest content active composition was close to obvious differences compared with that in control group ($P = 0.08$). 0.001%–0.01% paraquat could improve the activities of CAT, POD, and PPO, while 0.1% paraquat could decrease their activities [Figures 1-3]; the contents of schizandrin, deoxyschizandrin, and schisandrin B were increased along with the increase of paraquat, but contents of these constitutes in 0.001%–0.01% paraquat group were significantly lower than that in control group, and that in 0.1% paraquat group was slightly higher than that in control group [Figure 4a and 4b].

For ROS scavenger, 1–10 mmol/l propyl gallate could decrease the activities of CAT, POD, and PPO, which was negatively correlated with the oxidase activity [Figures 1-3], and the contents of schizandrin, deoxyschizandrin, and schisandrin B had no significant changes [Figure 4a and 4b].

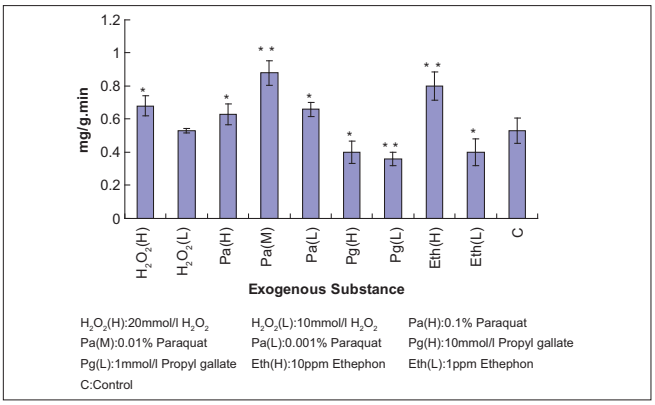

Figure 1: Effect of H_2O_2, paraquat, propyl gallate, and ethephon on CAT activity. Groups of data were compared with an analysis of variance followed by multiple comparison tests. **P* < 0.05 versus control group; ***P* < 0.01 versus control group

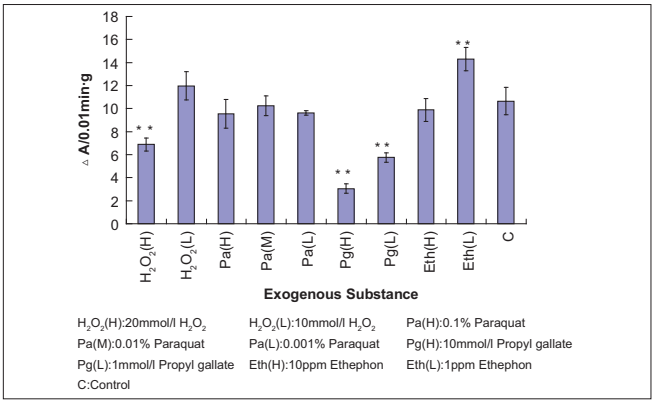

Figure 2: Effect of H_2O_2, paraquat, propyl gallate, and ethephon on peroxidase activity. Groups of data were compared with an analysis of variance followed by multiple comparison tests. **P* < 0.05 versus control group; ***P* < 0.01 versus control group

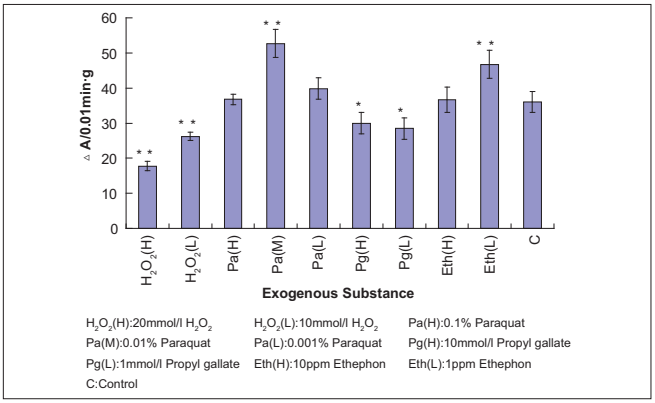

Figure 3: Effect of H_2O_2, paraquat, propyl gallate, and ethephon on polyphenol oxidase activity.Groups of data were compared with an analysis of variance followed by multiple comparison tests. **P* < 0.05 versus control group; ***P* < 0.01 versus control group

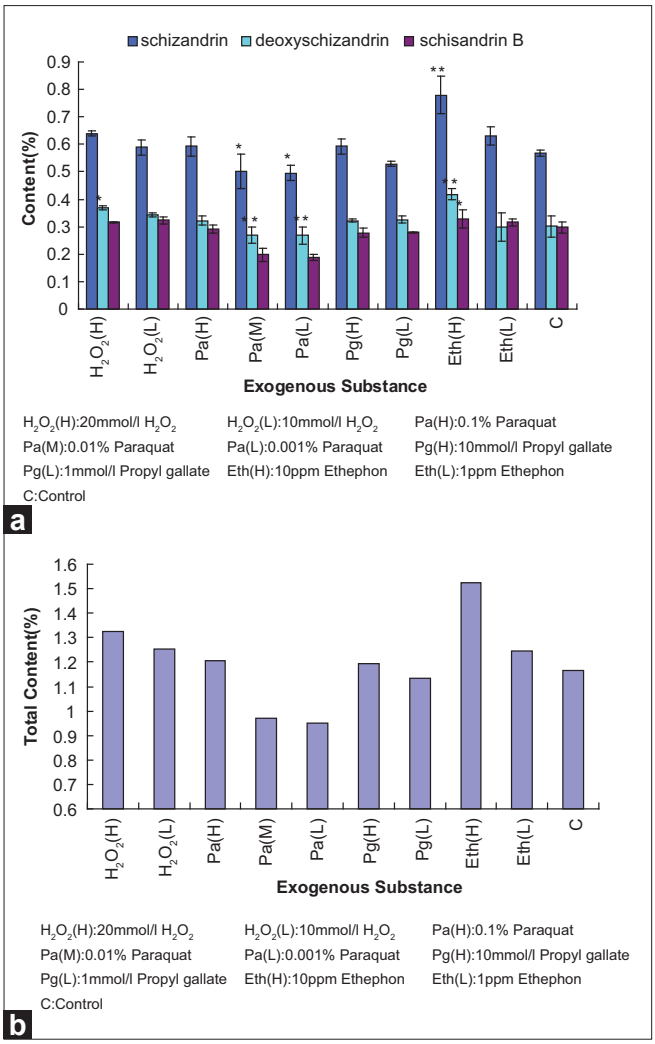

Figure 4: (a) Effect of H_2O_2, paraquat, propyl gallate, and ethephon on contents of schisandrin, deoxyschizandrin, and schisandrin B. Groups of data were compared with an analysis of variance followed by multiple comparison tests. **P* < 0.05 versus control group; ***P* < 0.01 versus control group. (b) Effect of H_2O_2, paraquat, propyl gallate, and ethephon on total contents of schisandrin, deoxyschizandrin, and schisandrin B

For ROS inductive agent ethephon, within 1–10 ppm range, POD activity was increased, and PPO activity was decreased along with the increase of ethephon. The contents of schizandrin, deoxyschizandrin, and schisandrin B were remarkably increased along with the increase of ethephon [Figure 4a and 4b].

DISCUSSION

Under ecological stress such as drought dehydration, salt damage, cold injury, heat shock, heavy metal pollution, nutrition deficiency, ultraviolet radiation, pathogenic bacterium infestation, and intense light,[10,11] the homeostasis between absorption and consumption of light energy by fixing CO_2 in chloroplasts of plant cells is broken, resulting in excessive light energy absorption. Meanwhile, O_2 generated from photosynthesis is accumulated due to stomata closure regulated by abscisic acid under stress, and then O_2 is reduced into O_2^- (Mehler reaction). It has been proved that the prime characteristic of plant cell under stress is to produce reactive oxygen species (ROS)[12] In mitochondrion, about 2%–3% of electrons are leaked out from respiratory chain enzyme complex I and III, resulting in more O_2 being reduced into O_2^-,[13] further transformed into $OH^•$, H_2O_2, $O^•_2$, NO, etc., which induce a variety of physiological and metabolic changes. In recent years, plenty of studies revealed an intensive relation between ROS and secondary metabolism that the contents of constitutes deriving from secondary metabolism increasing along with ROS contents under stress. As we know, ROS as signaling molecule in various levels from plasma membrane to the nucleus could serve as the messenger to participate in metabolic pathways, and affect secondary metabolism in different processes of gene expression, synthesis and activity of enzyme, various enzymatic reaction, etc.[13] (1) Promotion of gene expression. It is the most prevalent signal transduction pathway via phosphorylation and dephosphorylation of protein kinases and protein phosphatase on target proteases to promote related gene expression. (2) Activation of Ca^{2+} channel. Activation of Ca^{2+} channel in plasma membrane by ROS can cause an intracellular increase of Ca^{2+}, which mediate phosphorylation and dephosphorylation of protease, as well as induce relevant genetic expression. Many credible evidences have been obtained from plant adjustment reaction to low temperature, drought, and salt stress. (3) Modification of protein structure. ROS can modify protein structure and function through disulfide bond formation and cleavage, and it has been proved that the increase of intercellular ROS can raise some enzyme activity. (4) Direct or indirect actions to transcription. ROS begins to change at first according to the time sequence, the metabolism modification are carried out only in ROS-enzyme-metabolism order in the light of chemical reaction direction, therefore, from cause-and-effect relationship, ROS can be regarded as a headstream of secondary metabolic regulation, or an intermediary between ecological stress and secondary metabolism.

The ROS regulators, different from ecological factors' continuous affection on plants, can create a great fluctuation on ROS levels. Therefore, relatively stable antioxidase activity was chosen as an indicator to judge ROS levels. There are two kinds of consequences after the increase of ROS in plant: (1) Hypo-concentration ROS, as signal molecule, can activate adaptative and defensive response.[14,15] In the initial stage, antioxidases such as superoxide dismutase (SOD), CAT, and antiscorbic acid oxidase (APX) are inhibited to implement signal

messenger action of ROS, then those enzyme activities increase to eliminate ROS damage and to restore low-level homeostasis.[14] Paraquat is an effective and widely used herbicide which can produce ROS in plant tissues or organs, after paraquat was sprayed on leaves of *S. chinensis*, except POD, the activities of CAT, SOD, and PPO were significantly enhanced to remove excessive ROS, but failed to increase the bioactive consitituents of *S. chinensis* [Figure 4a and 4b]. Propyl gallate, after every day for a month spray, could effectively decrease ROS in plant, but lower levels of ROS lead to lower activities of oxidases at the same time, indicating that propyl gallate fails to eliminate inherent ROS for a long period, and relative high level of ROS still lead to high outputs of secondary metabolites (2). Hyper-concentration ROS, as injurious factors, could aggravate cell and organism damage. The activity of NADPH oxidase, peroxisomes, and oxidases in cell wall could be enhanced by hypso-concentration ROS, which will produce more ROS. Meanwhile, the activities of CAT, POD, and APX, as well as the levels of antioxidants, such as ascorbic acid and glutathione were decreased, which disturb the homeostasis between ROS and antioxidant system and result in a huge accumulation of ROS.[13] After sprayed every day for a month, 10–20 mmol/l H_2O_2 could obviously decrease the activities of POD and PPO, and break the homeostasis between ROS and secondary metabolism, which indicated that excessive ROS could possibly increase secondary metabolites [Figure 4a and 4b]. According to the above survey, it is necessary to improve usage amount of exogenous substance.

Various metabolic pathways may exist between environmental stresses and secondary metabolism, amounts of in-depth studies have focused on ethylene and pointed out that the correlation between environmental stresses and secondary metabolism is realized by ethylene changes. It has been proved that O_2^- could improve the formation and accumulation of ethylene, which results in a dramatic increase of membrane permeability[16,17] and various secondary metabolisms. Ethylene can induce the formation of resin ducts of *Liquidambar formosana* and raise the contents of volatile oil.[18] A certain concentration of ethephon stimulated 8 years old of *Santalum album* to produce sandalwood in the short term.[19] Wind can promote the production of ethylene to strengthen the intensity of the stem and avoid hazards, with no volatile oil in the 10 years old *S. album* heartwood undamaged by wind, but 1/2 volatile oil of the 25 years old one in the same age damaged by wind.

The carriers of ROS such as the H_2O_2 and paraquat increased the contents of secondary metabolites lower than inductive agent of ROS such as ethephon; however, propyl gallate as a ROS scavenger did not decrease the contents. At the upstream of plant metabolic regulation, even if moderate concentration of exogenous substance are used to increase or decrease ROS for long periods, ROS remains in a relatively steady state finally due to correspondingly regulation of oxidase in *S. chinensis* under complicated regulation systems, indicating that plants can adapt to the environment well under moderate stresses. But at the hypso-concentration ROS condition, oxidase activities are obviously decreased and the system homeostasis for metabolic regulation are broken, which raise the outputs of secondary metabolites in plants. At middle stream, moderate concentration of exogenous substance from ROS can avoid the regulated effects of metabolism systems, and increase secondary metabolites. This investigation can provide initial evidence for ROS in regulation of secondary metabolite output. Therefore, further experiment may be necessary to establish a wider application prospect of the study on the metabolic homeostasis system and oxidase inhibitor.

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