Reactive Oxygen Species Scavenging Activity of Jixueteng Evaluated by Electron Spin Resonance (ESR) and Photon Emission

Toshizo Toyamaa, Satoko Wada-Takahashib, Maomi Takamichib, Kiyoko Watanabee, Ayaka Yoshidab, Fumihiko Yoshinoh, Chihiro Miyamotob, Yojiro Maehatab, Shuta Sugiyamab, Shun-suke Takahashib,*
Kazu ToDod, Masaichi-Chang-il Leea and Nobushiro Hamaday

†These authors contributed equally to this work.
takahashi.shunsuke@kdu.ac.jp

Received: August 29th, 2014; Accepted: October 20th, 2014

Jixueteng, the dried stem of Spatholobus suberectus Dunn (Leguminosae), is a traditional Chinese herbal medicine that is commonly classified as a herb that promotes blood circulation and can be used to treat blood stasis. The aim of this study was to examine the reactive oxygen species (ROS) scavenging activity of Jixueteng and other herbal medicines. The ROS scavenging activities of the water extracts of Jixueteng, Cnidium officinale and Salvia miltiorrhiza were examined using an electron spin resonance (ESR) technique and faint luminescence measurement. The ESR signal intensities of the superoxide anion (O_2•−), hydrogen peroxide (H_2O_2) and hydroxyl radical (HO•) were reduced more by Jixueteng than the other herbal medicines we tested. High photon emission intensity to hydrogen peroxide (H_2O_2) and HO• was observed in Jixueteng using the XYZ chemiluminescence system that was used as faint luminescence measurement and analysis. The results of the present study revealed that the ROS scavenging activity of 8% Jixueteng was the strongest among the herbal medicines we tested. It has been reported that Jixueteng includes various polyphenols. In the ROS scavenging activity by Jixueteng, it is supposed that the antioxidant activity caused by these polyphenols would contribute greatly. In conclusion, a water extract component of Jixueteng had potent free radical scavenging activity and an antioxidative effect that inhibited the oxidative actions of O_2•−, H_2O_2 and HO•. Therefore, Jixueteng represents a promising therapeutic drug for reactive oxygen-associated pathologies.

Keywords: Jixueteng, Herbal medicine, Reactive oxygen species scavenging activity, XYZ chemiluminescence system, Electron spin resonance

Jixueteng is a herbal medicine that possesses pharmacological properties, such as improving the circulation, analgesia, and increasing the number of white and red blood cells, and is composed of the dried stems of Spatholobus suberectus Dunn and Millettia dielsiana Harms, both family Leguminosae [1-5]. We recently reported the potent local anti-infection effects of Jixueteng on oral indigenous bacteria and also demonstrated that it inhibited alveolar bone loss [6,7]. These findings suggested that Jixueteng may be used as a safe and effective therapeutic agent for periodontal disease through various approaches because of its antibacterial and immune activities, in addition to its ability to improve the circulation. However, Jixueteng has not yet been clinically applied to the oral field, and its effects on reactive oxygen species (ROS) in inflamed regions and the detailed mechanisms underlying these pharmacological actions remain unclear. ROS play key roles in various physiological and pathological events. The overproduction of ROS can cause oxidative damage to biomolecules, (lipids, proteins, and DNA), which ultimately results in many chronic diseases in humans such as atherosclerosis, cancer, diabetes, rheumatoid arthritis, post-ischemic perfusion injury, myocardial infarction, cardiovascular diseases, chronic inflammation, stroke, septic shock, aging, and other degenerative diseases [8,9]. In the present study, we measured the ROS scavenging activity of Jixueteng using X-band electron spin resonance (ESR) spectroscopy and the XYZ chemiluminescence system. This XYZ chemiluminescence system is an assay that is used to detect the energy that is emitted as ultra-weak luminescence when ROS are eliminated by a scavenger. This chemiluminescence system suggests that the two species of a hydrogen donor (Y) and a mediator or accelerator (Z) are important and necessary to complete the elimination of ROS (X) through light energy transformations. In this system, the mediator (Z) represents the ability of a compound to mediate the transfer of hydrogen or an electron between X and Y [10]. This system is characterized by a photon detection method that uses fixed XY reagents to search for Z species, XZ reagents for Y species, and YZ reagents for X species. ROS scavenging activity correlates with luminescence intensity; therefore, this luminescence system differs from chemiluminescence, which is characterized by a reaction between luminol and hydrogen peroxide (H_2O_2), and also other methods such as the ORAC and DPPH assays. Based on this evidence, the chemiluminescence system has been applied to screen for ROS scavenging activity and the freshness of foods and agricultural products [11-13]. The ROS scavenging activity of Jixueteng has also been confirmed by X-band ESR spectroscopy. The aim of this study was to investigate the ROS scavenging activity of Jixueteng and the possibility of this herbal medicine being an effective therapeutic agent for ROS-related pathologies.
Evaluation of ROS scavenging activities of Jixueteng and other herbal medicines by ESR: The Jixueteng water extract markedly reduced the ESR signal intensity of HO• in a concentration-dependent manner from 0.025% to 2% (Figure 1A). The maximum scavenging activity was observed at 2%, and was equivalent to the activity of 10 mM deferoxamine (Figure 1B).

The HO• scavenging activities of the water extracts of Cnidium officinale, Salvia miltiorrhiza and CoQ10 were also concentration-dependent, while that of Jixueteng was significantly higher than the 2% (P < 0.001) and 4% (P < 0.05) water extracts of CoQ10 (Figure 2).

The Jixueteng water extract also markedly reduced the ESR signal intensity of O2• (Figure 3A) in a concentration-dependent manner from 0.25 to 8%. The maximum scavenging activity was noted in the 2% Jixueteng water extract, and was equivalent to the activity of 100 U/mL SOD (Figure 3B). The O2• scavenging activities of the water extracts of Cnidium officinale, Salvia miltiorrhiza and CoQ10 were also concentration-dependent. The O2• scavenging activities of Salvia miltiorrhiza and Jixueteng were significantly higher than those of CoQ10 (P < 0.001). The scavenging activity level of the 8% water extract of Salvia miltiorrhiza was similar to that of the 8% water extract of Jixueteng (Figure 4).

Evaluation of ROS scavenging activities of Jixueteng and other herbal medicines using XYZ chemiluminescence: H2O2 and HO• scavenging activities were detected in the water extracts of Jixueteng, Cnidium officinale and Salvia miltiorrhiza; gallic acid was used as a reference compound of an antioxidative reagent on XYZ chemiluminescence (Figures 5, 6). The water extracts of each herbal medicine exhibited potent H2O2 and HO• scavenging activities in a concentration-dependent manner (A of Figures 5 and 6). The ROS scavenging activity of the 8% water extract of Jixueteng was the strongest among the herbal medicines we tested (B of Figures 5 and 6).

The Jixueteng, which is listed in the Chinese Pharmacopoeia, is the dried stem of Spatholobus suberectus Dunn of the family

**Figure 1**: Effects of 0.025-8% Jixueteng water extracts on the ESR spin adduct of HO• (A) and the effects of deferoxamine on the ESR spin adduct of HO• (B). X-band ESR was used to determine the signal decay of DMPO-OH.

**Figure 2**: Relative inhibition rate of various herbal medicines to the HO• signal intensity. Jixueteng: closed circle, Cnidium officinale: open circle and Salvia miltiorrhiza: closed square. Data are presented as the mean ± SD. (n = 5 in each group). **P < 0.001, *P < 0.05 vs CoQ10 (as control data: open square).

**Figure 3**: Effects of 0.025-8% Jixueteng water extracts on the ESR spin adduct of O2• (A) and the effects of 0.01-1.000 U/mL SOD on the ESR spin adduct of O2• (B). X-band ESR was used to determine the signal decay of DMPO-OOH.

**Figure 4**: Relative inhibition rate of various herbal medicines to the O2• signal intensity. Jixueteng: closed circle, Cnidium officinale: open circle and Salvia miltiorrhiza: closed square. Data are presented as the mean ± SD. (n = 5 in each group). **P < 0.001, *P < 0.05 vs CoQ10 (as control data: open square).

Figure 5: Effects of herbal medicines on photon emission (A) and photon emission intensities (B) in the presence of the oxidant H2O2. The reaction mixture contained X (250 μL), Y (100 μL) and Z (250 μL) reagents in a 12-well plate (A). The color bar in (A) shows light emitting intensity that is antioxidant activity, and the red in top of color bar shows the highest antioxidant activity. Luminescence was initiated by adding the concentration-fixed XZ mixture into various concentrations of the Y solution. These experiments were performed in the presence of the oxidant H2O2 (X) + a hydrogen donor, an 8% water extract of a herbal medicine (Jixueteng, Cnidium officinale and Salvia miltiorrhiza) or 5 mM gallic acid (Y) + mediator (Z) (antioxidant activity donor, an 8% water extract of a herbal medicine (Jixueteng, Cnidium officinale and Salvia miltiorrhiza) or 5 mM gallic acid (Y) + mediator (Z).
Leguminosae. However, more than 30 plant species have been used under the general name “Jixueteng” in traditional Chinese and folk medicine [1,2]. Jixueteng is a traditional Chinese medicine that improves, increases, and tonifies blood circulation [3], and is also used to treat irregular menstruation, blood deficiencies, and rheumatalgia [4,5]. The chemical profile of Jixueteng was investigated extensively by Tang et al. [14]. Jixueteng also contains various components that are predominantly polyphenols, including flavonoids [15, 16]. Previous chemical and pharmacological investigations have indicated that flavonoids are the main ingredients of Spatholobus suberectus [17, 18]. The other herbal medicines that were used as a reference for Jixueteng in the present study, Salvia miltiorrhiza and Cnidium officinale, have been used to tonify the blood and increase its circulation, relieve rigidity of muscles and joints, and promote menstruation. Therefore, these herbal medicines improve blood circulation and invigorate blood in a similar manner to Jixueteng.

Several assays have been frequently used in clinical studies to estimate antioxidant capacities, including the oxygen radical absorption capacity (ORAC) [19, 20] and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays [21, 22]. The ORAC assay determines fluorescence decay resulting from the oxidation of fluorescein by the peroxy radical. An antioxidant suppresses fluorescence decay. Thus, the ORAC assay is an indicator of antioxidant activity. The DPPH assay measures changes induced in color by the antioxidant material using the stable radical DPPH. Previous studies using these methods demonstrated that the antioxidant activity of Jixueteng (Spatholobus suberectus) was high [20, 22]. Methods that are frequently used to evaluate antioxidant activity only determine the antioxidant effect for Y in the XYZ system. However, in the XYZ photon emission system, Z (a mediator or accelerator) facilitates Y antioxidant activity only determine the antioxidant effect for Y in the XYZ system. Therefore, antioxidant activity of Y will be more stable in the presence of Z [10]. In other words, an evaluation of antioxidant activity using this XYZ system considers not only antioxidant activity, but also the stability of the antioxidant substance.

In the present study, we investigated the antioxidative effects of Jixueteng and other herbal medicines, Cnidium officinale and Salvia miltiorrhiza, using ESR spectroscopy and ultra-weak photon detection based on the XYZ chemiluminescence system. This system does not employ chemiluminescence reagents such as luminol, which reacts with hydrogen peroxide to produce luminescence, the intensity of which is dependent on ROS scavenging activity [10]. Okubo et al. recently reported that natural radical scavengers produce ultra-weak light emission (photon emission) in the presence of active oxygen species and acetaldehyde [10]. This photon emission occurred nonenzymatically without photon enhancement, such as that observed with luminol. We demonstrated that photon emission was observed due to the antioxidant activities of several traditional Chinese medicines. However, in the XYZ system, antioxidants continued receiving energy from ROS. If this antioxidant substance affects under conditions by which the known antioxidant activities of several traditional Chinese medicines are measured, it is possible that photon emission is observed. Although the ROS (X) has strong reactivities, it did not exhibit photon emission. Therefore, ROS is an intermediate state of energy. By adding an ROS scavenger (Y), for example polyphenols such as flavonoids, anthocyanins, and catechins and also traditional Chinese medicines extracted from leaves, root, and bark, an excited-state compound, a Y’-radical, will be produced. Y’-radicals accumulate at this point because the X and Y mixture does not exhibit photon emission. The release of energy is observed following the addition of a receptive species, a mediator or accelerator (Z), such as acetaldehyde and saponins. This energy release represents photon emission in the XYZ system. The Y’-radical changes to a stable state with the oxygenation or degradation of Z by a radical reaction. Therefore, the mechanism responsible for the scavenging of ROS by flavonoids can be deduced from the photon emissions observed using this system composed of X, Y, and Z [10-13].

We demonstrated that the Jixueteng water extract had strong H2O2, HO• and O2•− scavenging activities. These ROS are known to be closely associated with various pathologies in the body (inflammatory reactions, ischemic cytopathy, adverse effects of drugs and cancer) and aging. The ROS scavenging activity of Jixueteng was markedly stronger than that of the other herbal medicines, and the effects of the 8% water extract of Jixueteng were similar to those of an iron chelator, 10 mM deferoxamine, and 100 μM of the O2•− scavenger, SOD. When its antioxidative effects on H2O2 were evaluated using the XYZ theory, the antioxidant activity of the 8% water extract of Jixueteng was higher than that of 5 mM (0.085%) gallic acid, and was also the strongest among the herbal medicines we tested. Gallic acid is a polyhydroxyphenolic compound that can be found in various natural products, such as green tea, grapes, strawberries, bananas, and many other fruits [23]. This compound is widely used as a natural antioxidant for the oils and fats of food, cosmetics, and pharmaceutical products. Gallic acid has also been shown to inhibit significantly cell proliferation, induce apoptosis in a series of cancer cell lines, and exhibit selective cytotoxicity against tumor cells with higher sensitivity than normal cells [24].

Many herbal medicines are known to possess some degree of antioxidant activity, and phenolic compounds mainly contribute to this activity [20]. Although we did not investigate the involvement of phenolic compounds, there is no doubt that certain water-soluble components involve polyphenolic compounds [14-16]. Therefore,
the water extract components involved in this reactive oxygen scavenging activity need to be identified and their relationship with the pharmacological effects of Jixueteng investigated; this will form part of our future studies. In the present study, the ROS scavenging activity of Jixueteng was almost constant at concentrations of more than 2%. Therefore, further antioxidative effects would not be obtained. However, since phenolic compounds, including those in Jixueteng, may possess the properties of both antioxidants and prooxidants under some conditions [25], cytotoxicity should be evaluated at concentrations of more than 8%. The water extract component of Jixueteng was shown to have potent free radical scavenging activity and antioxidative effects that inhibited the oxidative actions of H2O2, O2•−, and HO•. Therefore, Jixueteng represents a promising therapeutic drug for reactive oxygen-associated pathologies.

Experimental

Preparation of herbal medicine water extracts: The dried vines of Spatholobus subercoccus Dunn, as well as the herbal medicines, Salvia miltiorrhiza and Cnidium officinalis (Tochimotoenpakuo, Osaka, Japan), were used in the present study. Each sample (200 g) was extracted by heating at 95°C in 1 L of distilled water for 3 h, followed by filtration (Toyo filter paper No.2) to remove debris. All samples were finally designated as 20% water extracts. Each extract solution was cooled to room temperature, diluted with phosphate-buffered saline (PBS, pH 7.0), and subjected to experiments. To compare the ROS scavenging activities of the herbal medicines, either antioxidative reagents, gallic acid (Wako Pure Chemical Industries, Osaka, Japan), or ROS scavengers, deferoxamine (Novartis Pharma AG, Basel, Switzerland), SOD (Sigma-Aldrich, St. Louis, MO, USA), and coenzyme Q10 (CoQ10: Sigma-Aldrich, St. Louis, MO, USA), were used.

Determination of ROS scavenging activities of Jixueteng and other herbal medicines using ESR: ESR spin trapping was conducted with a ROS generating system containing 5,5-dimethyl-1-pyrroline-N-oxide (DMPO: Dojindo Laboratories, Kumamoto, Japan).

The hydroxyl radical (HO•) was generated by the Fenton reaction (H2O2 plus FeSO4), as described previously [26, 27]. To measure HO•, 160 μL of phosphate-buffered saline (PBS, pH 7.0), 10−3 M FeSO4 (Sigma-Aldrich, St. Louis, MO, USA), 25 μL of the water extract of the test herbal medicine at specific concentrations (0.02, 0.2, 2, 4, or 8%) (Jixueteng, Cnidium officinale, Salvia miltiorrhiza) or PBS for the condition without herbal medicine, and 15 μL of 8.8 M DMPO were mixed and reacted with 25 μL of 10−4 M H2O2 (Wako Pure Chemical Industries, Osaka, Japan). This reaction solution was aspirated into a 130 μL ESR flat cell after 1 min, and ROS scavenging activities were measured using ESR. Deferoxamine and CoQ10 were used as reference compounds for the antioxidative reagents. This substance has been used in other studies [26, 27]. All experiments were repeated a minimum of 4 times.

Determination of ROS scavenging activities of Jixueteng and other herbal medicines using the XYZ chemiluminescence method: The ROS-generating substances, 0.6% H2O2 (Wako Pure Chemical Industries, Osaka, Japan) and/or HO• (0.6% H2O2 + 25 mM FeSO4) were used for X. Herbal medicine water extracts from 2 to 8% were used as ROS scavengers: Y and 10% acetaldehyde/saturated potassium bicarbonate were used as the mediator: Z. Chemiluminescence was detected using a photon counting charge-coupled device (CCD) camera (C2400-30, Hamamatsu Photonics, Japan) connected to an imaging PMT (a photocathode and microchannel plates), and a position-sensitive detector coupled to an ARGUS-20 (Hamamatsu Photonics, Japan) and/or PBS for the condition without herbal medicine, and 15 μL of 8.8 M DMPO were mixed and reacted with 25 μL of 10−4 M H2O2 (Wako Pure Chemical Industries, Osaka, Japan). The wave range of the detector was 360-650 nm. The reaction mixture was composed of X (250 μL), Y (100 μL), and Z (250 μL) reagents. Luminescence was initiated by adding the concentration-fixed XZ mixture into various concentrations of the Y solution in a 12-well plate. The ultra-weak light that was emitted was measured and accumulated every 10 sec, and this was repeated 200 times. These results were expressed as the emission intensity. ROS scavenging activity was evaluated by analyzing the emission intensity (ROS scavenging activity became stronger as the emission intensity increased). Gallic acid was used as a reference compound for the antioxidative reagents. This substance has been used in similar chemiluminescent studies as a reference for antioxidative effects [10-13]. All experiments were repeated 3 times.

Statistical analysis: An analysis of variance and multiple comparison tests using the Tukey’s method were applied to determine differences among the herbal medicine groups. The Student’s unpaired t-test was used when comparing one pair only. Data are expressed as the mean ± SD.

Acknowledgments - This work was supported, in part, by JSPS KAKENHI Grant Number 25460704 for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan. We dedicate this article to the memory of Dr. Yusuke Takahashi, our colleague and friend, who died in May, 2009.
References


