Chlorogenic acid in the oxidative stress injury triggered by Shuang-Huang-Lian injection

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Abstract. Injections of Chinese herbs are a novel approach to prepare traditional Chinese medicines. However, as injections of Chinese herbs have been extensively used, adverse drug reactions (ADRs) have been on the increase. Additionally, the mechanism for injections of Chinese herbs remains unclear. This study explored the potential role played by chlorogenic acid (CGA) in initiating oxidative stress injury triggered by the utilization of injections of Chinese herbs and the underlying mechanism. A total of 90 male Wistar rats were raised for varying periods by using Shuang-Huang-Lian (SHL) injection or CGA in diverse dosages. Western blot analysis examined the expression of nicotinamide adenine dinucleotide phosphate oxidase subunits, spectrophotometry was used to examine the activity taken by catalase, ELISA was used to examine the concentrations of inflammatory factors in serum, and intravital microscopy was employed to examine the microcirculation. The results showed that the excessive peroxide production induced by CGA in high-dose or SHL in the venule walls may well be through nicotinamide adenine dinucleotide phosphate oxidase along with a decline in the activity of catalase, and led to imbalance of basal levels of pro-(TNF-α) and anti-(IL-10) inflammatory cytokines. On the basis of the aforementioned results, the mechanism hidden behind the adverse effects of CGA induced by irrational use of Chinese herbal injection can be identified from a deeper perspective.

Introduction

The Chinese herbal injections are new preparations of novel approaches to prepare traditional Chinese herbal medicines designed within the past decade for convenience in practice in terms of practitioners and patients. However, as injections of Chinese herbs have been extensively used, there has been an increase in adverse drug reactions (ADRs), including drug-induced death, liver and kidney injury, shock, diarrhea, vomiting, asthma, erythra, and itchy skin (1-5). Such preparations, guided by a qualified practitioner of TCM, should have mild and low adverse effects, as previously documented (2). The majority of these ADRs however, correlate with irrational use especially overdose (2,6,7). In recent years, increasing attention has been paid to the mechanisms for ADRs. Nevertheless, little information is available on the constituent complexity of these injections.

Chlorogenic acid (CGA) is a ubiquitous component in most Chinese herbal injections, also usually employed as a typical marker to control the quality of TCM (8), such as Shuang-Huang-Lian (SHL) injection (according to the Pharmacopeia of China, 2005). This field remains problematic (9,10) though the allergenicity has constantly counted as the primary factor leading to adverse effects of CGA (11,12). On the other hand, CGA in lower concentrations eliminated hydroxyl radical and superoxide in vitro although CGA at higher concentrations produced radicals and served as a pro-oxidant, as previously reported (13). Oxidative damage to normal cells is likely to be one of the mechanisms of such drug-induced adverse reaction, which may be a good interpretation for why herbal injection-induced ADRs occur in the case of overdose more often than not (6). Nevertheless, which insults are bound by the adverse effects triggered by CGA remain unclear.

The present study determined the functions taken on by CGA in the oxidative stress injury triggered by injections of Chinese herbs by controlling SHL injection in different doses, i.e., the most frequently used and ADRs reported on Chinese herbal injections (14), and equivalent dose of CGA, respectively. The experimental flow diagram is shown in Fig. 1.
Materials and methods

Animals and reagents. A total of 90 male Wistar rats with a weight ranging 200-220 g were obtained from the Animal Center, Health Science Center, Peking University (Beijing, certificate no. SCXK 2006-0008). The rats were caged as per a 12-h light/dark circulation 40±5% of humidity and at 22±2˚C, and given standard water and diet without advance preparation. Prior to the experiment, the rats were fasted for 12 h. The animal model preparation, under the guidance of Animal Research Committee of Peking University, complied with the EU adopted Directive 2010/63/EU. Experimental Animal Ethics Branch subordinated by Biomedical Ethics Committee governed by Peking University approved the experiment protocols (LA2011-38). The study was also approved by the Ethics Committee of the TCM Hospital of Shijiazhuang Affiliated to Hebei University of Chinese Medicine (Shijiazhuang, China).

CGA was dissolved in sterile 0.9% normal saline, which was obtained from Sigma - Aldrich: Merck KGaA (St. Louis, MO, USA) with a purity ≥98%. SHL lyophilized powder injection (24 ampoules, 1.2 g/ampoule) was obtained from Heilongjiang Songhuajiang Pharmaceuticals Co., Ltd. (Harbin, China) also dissolved in 0.9% normal saline.

Drug administration and experimental groups. This study randomly split the rats into five groups according to weight. Additionally, the high-dose SHL injection [H-SHL; 420 mg/kg, 8 ml/kg/h, intravenous (i.v.) drip injection], high-dose CGA (H-CGA; 7 mg/kg, 8 ml/kg/h, i.v. drip injection), low-dose SHL injection (L-SHL; 20 mg/kg, 8 ml/kg/h, i.v. drip injection), low-dose CGA (L-CGA; 0.336 mg/kg, 8 ml/kg/h, i.v. drip injection) and normal saline (control, 8 ml/kg/h, i.v. drip injection) were given within 1 h via the left catheter of jugular vein, respectively. The body weight CGA with the concentration of 0.336 mg/kg was selected as a low dose as being a mean dosage of CGA in injection of Chinese herb being most frequently adopted. In addition, the adverse effect relative to this dose has been rarely reported. The dose recommended in the instructions of Qingkaling injection, an injection of traditional Chinese medicine, takes up merely 1/6 of the dose 7 mg/kg body weight. Additionally, the majority of the found adverse effects occur close to this dose (15). The L-SHL and the H-SHL group contained the same amount of CGA (0.336 and 7 mg/kg, according to the Pharmacopoeia of China, 2005) as L-CGA or H-CGA group, respectively. This study administered animals in another isolated experiment series by adopting saline or the drug whereby intraperitoneal bolus injection QD was for 7 or 14 days. Furthermore, the relevant examinations on the animals were carried out. Table I lists the animal number in line with the groups for the experiment.

Microcirculatory observation. Surgery was performed, complying with the previous descriptions (16). This study used intramuscular injection of 20% urethane (1 ml/100 g BW), anesthetizing the rats. The abdomen of rats was opened via a 25 to 30 mm cut. The ileocecal junction of the mesentery was gently exposed (10-15 cm caudal) outside. On that basis, the exposed junction was mounted on a plastic transparent stage for rats. Through continuous superfusion, the mesentery was preserved moist and warm at 37˚C with saline solution. An inverted microscope (DM IRB; Leica Microsystems GmbH, Cologne, Germany) was used to observe the microcirculation of mesentery via the objective lens (x20), and a 12 V, 100 W, direct current-stabilized light source was used to transilluminate the mesentery. A video camera with vibrant color (JK-TU53H; Toshiba, Tokyo, Japan) was installed on the microscope to capture the images from the microscopic angle. Subsequently, the image was transmitted onto a monitor (J2118A; TCL, Huizhou, China). A digital video disk video cassette recorder (DVR-R25; Malata, Xiamen, China) recorded the images. Single and unbranched venules (30-50 µm in diameter; 200 µm in length) were selected for the study (16).

After the observation of baseline (10 min), microcirculation was examined. For monitoring oxidant stress in the venule walls, 5 min before observation, topical dihydrorhodamine oxidant-sensitive, 123 fluorescent probe was applied to the surface of mesentery (10 µmol/l) (DHR; Molecular Probes: Thermo Fisher Scientific, Inc., Eugene, OR, USA). A fluorescence microscope inverted for 455 nm excitation light (DM IRB; Leica Microsystems GmbH), to record the fluorescence image 60 and 120 min after infusions at baseline.
**Table I. No. of animals for different experimental groups and various parameters.**

| Groups | Oxidant stress | Western blotting, ELISA and biochemical examination | Total |
|--------|----------------|---------------------------------------------------|-------|
| 2 h    |                |                                                   |       |
| Control| 6              | -                                                 | 6     |
| L-CGA  | 6              | -                                                 | 6     |
| L-SHL  | 6              | -                                                 | 6     |
| H-CGA  | 6              | -                                                 | 6     |
| H-SHL  | 6              | -                                                 | 6     |
| Day 7  |                |                                                   |       |
| Control| -              | 6                                                 | 6     |
| L-CGA  | -              | 6                                                 | 6     |
| L-SHL  | -              | 6                                                 | 6     |
| H-CGA  | -              | 6                                                 | 6     |
| H-SHL  | -              | 6                                                 | 6     |
| Day 14 |                |                                                   |       |
| Control| -              | 6                                                 | 6     |
| L-CGA  | -              | 6                                                 | 6     |
| L-SHL  | -              | 6                                                 | 6     |
| H-CGA  | -              | 6                                                 | 6     |
| H-SHL  | -              | 6                                                 | 6     |
| Total  | 30             | 60                                                | 90    |

The same animals from each group were used for the determination of oxidant stress. For western blotting, ELISA and biochemical examination, tissues were collected from the same animals in each group. 2 h, one injection; day 7, 7 injections; day 14, 14 injections; L-CGA, low-dose CGA; L-SHL, low-dose SHL; H-CGA, high-dose CGA; H-SHL, high-dose SHL.

Additionally, this study employed Image-Pro Plus 5.0 software to, respectively, measure the extravascular interstices (Ie) and fluorescence intensity of walls venule (Iv). How Ie and Iv were different from each other was ascertained for each point of time. Besides, the ratio of each value calculated to the baseline (16).

**ELISA analysis.** After injections for 7 or 14 days, the blood was extracted from a branch of the descending aorta of the rats. For measuring the concentrations of tumor necrosis factor-α (TNF-α) and interleukin-10 (IL-10), 1 ml arterial systemic serum was collected. Using ELISA kits from RapidBio Systems, Inc. (Carlsbad, CA, USA), the concentrations of TNF-α (pg/ml) and IL-10 (pg/ml serum) were ascertained. The assays were conducted as instructed by the manufacturer (17).

**Determination of activity of catalase.** After 7 or 14 days of injection, the serum was collected and the blood extracted from a branch of the descending aorta of rats. The activity by catalase (CGAT) was evaluated by exposing samples to excessive hydrogen peroxide with the purpose of decomposition. Additionally, to produce a comoles compound, was reacted with the ammonium molybdate with the residual hydrogen peroxide, which was absorbed at 405 nm to the greatest extent (18). The the antioxidase activities as U/ml serum were noted. Through commercial kits, the assessments of CGAT activity was performed (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). A bicinchoninic acid (BCA) protein assay kit was used to analyze the overall content of protein in samples (Sun Biomedical Technology Co., Ltd, Beijing, China) (18).

**Western blotting of Nox4, p22phox, p47phox protein expression.** The lung tissues, brain tissues and tissues of terminal ileum of rats were removed 7 or 14 days after injection. Tissues were homogenized and minced in lysis buffer on ice [pH 7.4, 0.1% sodium dodecyl sulfate (SDS), 1% Nonidet p40 (NP40) solution, 50 mM Tris/HCl, and 150 mM NaCl], and centrifuged for 10 min at 12,000 x g. The supernatant was isolated with cytosol proteins. BCA protein assay kit was used to study and quantify the overall protein in the homogenates (Sun Biomedical Technology Co., Ltd.). The prepared samples were boiled for 5 min in gel loading buffer [pH 6.8, 1% bromophenol blue, 1.56% dithiothreitol (DTT), 10% glycerol, 2% SDS, and 12.5 mM Tris/HCl]. The proteins (50 µg) were separated in equal amounts for each sample on a mini-gel of 10% SDS-polyacrylamide for 2 h at a constant voltage of 100 V. The proteins were transferred to membranes of polyvinylidene difluoride (PVDF) through electrophoresis for 16 h at 30 V. The membranes were blocked at room temperature in TBS-T in 5% (w/v) non-fat dry milk for 1 h (pH 7.4, 0.1 mM Tween-20, 100 mM NaCl, and 10 mM Tris/HCl). The membranes were cultured through using rabbit polyclonal IgG against β-actin (1:500), p47phox (1:200), p22phox (1:200) (all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), Nox4 [2 µg/ml; Abcam (Hong-Kong), Ltd., Hong-Kong, China] overnight. The samples were washed, and the membranes cultures for 2 h through the secondary antibodies conjugated by enzyme used in immunohistochemistry to label antigens and their antibodies (1:3,000; Santa Cruz Biotechnology, Inc.). Radiographic film was subsequent exposed and a chemiluminescence system was enhanced, and detected the antibody labeling. The optical density was visualized for the bands. Besides, the density was normalized to the density taken by β-actin (19).

**Statistical analysis.** Data were presented as the mean ± standard error (SEM). Additionally, SPSS 17.0 software for statistics was employed for analysis (SPSS, Inc., Chicago, IL, USA). Multiple comparisons were made using ANOVA followed by Tamhane’s T2 and LSD tests. This study accepted statistical significance at p<0.05.

**Results**

**Determination of intensity of fluorescence by DHR in the walls of venules.** The intensity of fluorescence by DHR in the walls of venules at diverse times in the five groups were determined. In rat mesenteric walls of venule, we failed to detect DHR fluorescence prior to infusing the five groups. Additionally, the low fluorescence intensity remained in the L-SHL, L-CGA and control group. Conversely, a marked DHR fluorescence was triggered by H-CGA in walls of the venules for 60-120 min as the groups were infused taking on evident...
difference in contrast with control group. Likewise, H-SHL induced an obvious DHR fluorescence at 120 min in contrast with control group and a little under the H-CGA group level, which indicated the inherent capacity by CGA and SHL to trigger reactive oxygen species (ROS) production at high dose from venules.

Analysis of IL-10, TNF-α and CGAT in rat serum. Inflammatory factor parameters in rat serum were recorded according to the experimental protocol. The changes of IL-10, TNF-α, IL-10/TNF-α and CGAT in rat serum at day 7 and 14 are shown in Figs. 2 and 3. H-SHL suppressed the concentration of IL-10 after seven injections compared with control. Furthermore, the expression of TNF-α was significantly higher in H-CGA and H-SHL group than that in control group at day 7, and no significant change was found in proinflammatory cytokines at day 14 point. Moreover, the ratio of IL-10 and TNF-α decreased in H-CGA group at day 7 and 14 as compared to control group, and, the ratio decreased in response to injection with H-SHL, which was similar to the change in H-CGA group at the two time points (Fig. 2).

The CGAT in the antioxidant principle pertaining to the organism was contained. As shown in Fig. 3, the activity of CGAT decreased in H-CGA and H-SHL group after seven injections. At day 14 time point, the activity of CGAT similarly decreased in the two groups. Other groups did not affect the activity of the enzyme during the observation.

Figure 2. Concentrations of IL-10 and TNF-α in rat serum. (A) IL-10 content (pg/ml); (B) TNF-α content (pg/ml); (C) the ratio of IL-10 to TNF-α. Data are expressed as mean ± SEM of six animals. *p<0.05 vs. control group (day 7), †p<0.05 vs. control group (day 14). IL-10, interleukin-10; TNF-α, tumor necrosis factor-α; L-CGA, low-dose CGA; L-SHL, low-dose SHL; H-CGA, high-dose CGA; H-SHL, high-dose SHL.

Discussion

Injecting SHL or CGA in high dose probably result in an imbalance between mechanism in antioxidant and oxidant, which was proven in this study in rats. Such injection triggers oxidant stress, inclusive of gained ROS production in wall of venules, and decreased activity of CGAT. Promoted expression of p47phox, p22phox and Nox4 was observed in response to the injection of SHL or CGA in high dose, indicating the containment of nicotinamide adenine dinucleotide phosphate oxidase in the CGA-triggered oxidant stress. Moreover, exposure to H-CGA or SHL undermined the balance of basal levels of pro-(TNF-α) and anti-(IL-10) inflammatory cytokines, manifested as decreased IL-10 to TNF-α ratio. As overdose drugs were added, adverse effects observed overall showed up.

For monitoring the oxidative stress, the probe DHR was used to ascertain extent of ROS in other types and intracellular H2O2 levels in other cell types and in incubated endothelial cells (20-22). As outcomes acquired in this report indicate, imbalance between the systemic manifestation of ROS in microcirculatory system was promoted in both groups of SHL and CGA taking on high dose starting from 60 and 120 min administration, respectively. However, injection with CGA
Figure 4. The protein expression of Nox4, p22phox and p47phox in the ileum tissues of rats. (A and E) Representative western blots of Nox4, p22phox and p47phox at day 7 and 14. The protein expression of (B and F) Nox4, (C and G) p22phox, and (D and H) p47phox at day 7 and 14. Lower columns: quantification of the western blotting results shown in (A and E). Data are expressed as means ± SEM of three animals. *P<0.05 vs. control group (day 7), #P<0.05 vs. control group (day 14). L-CGA, low-dose CGA; L-SHL, low-dose SHL; H-CGA, high-dose CGA; H-SHL, high-dose SHL.

Figure 5. The protein expression of Nox4, p22phox and p47phox in the lung tissues of rats. (A and E) Representative western blots of Nox4, p22phox and p47phox at day 7 and 14. The protein expression of (B and F) Nox4, (C and G) p22phox, and (D and H) p47phox at day 7 and 14. Lower columns: quantification of the western blotting results shown in (A and E). Data are expressed as means ± SEM of three animals. *P<0.05 vs. control group (day 7). L-CGA, low-dose CGA; L-SHL, low-dose SHL; H-CGA, high-dose CGA; H-SHL, high-dose SHL.
or SHL in low dose did not enhance the generation of ROS. This finding was consistent with previous research (23). CGA-triggered oxidative stress depends on the dose.

As the presented, CGA or SHL in high dose evidently declined the activity of antioxidase CGAT in rat serum after 7 and 14 day injections. An equilibrium between clearance and ROS production is of necessity for the standard cellular roles. As the antioxidant capacity of ROS is overcome by its cellular production, the equilibrium is broken, and an imbalance between the systemic manifestation of ROS shows up. CGAT counts as the primary antioxidase in cells produced by an enzyme and is of critical significance for offering protection against stress generated in oxidation (24). This antioxidase can be evidently depleted, and its activity is remarkably reduced as ROS is excessively accumulated. This finding conformed to the outcomes detected in terms of intensity of fluorescence relative to DHR (over ROS production). Accordingly, the injury by oxidant stress exerted by high-dose drug is probably triggered by dissipation of the antioxidase enzymes and ROS over production.

Scholars have always considered ROS over production as a pathological process charged with organ dysfunction and cellular damage (19). Given the necessity to take by nicotinamide adenine dinucleotide phosphate oxidase in producing stress related to oxidation of dysregulated vascular oxidation-reduction circumstance (25), we investigated nicotinamide adenine dinucleotide phosphate oxidase family members and explored the potential of the enzyme as the source of high dose CGA or SHL-induced ROS. It was found that H-CGA and SHL significantly increased Nox4, p22phox or p47phox protein expression in the terminal ileum, lung and brain tissues after 7 or 14 day injections. It was reported that Nox4 is present in all vascular walls and is significantly more abundant than any other Nox enzyme (26). The high expression of Nox4, p22phox and p47phox suggests that nicotinamide adenine dinucleotide phosphate oxidase plays a role in high dose CGA and SHL-induced ROS production especially derived from vascular walls. Even so, the possibility remains for the involvement of other peroxidases present and other mechanisms in vascular cells induced by CGA or SHL overdose.

This study observed a decrease of IL-10/TNF-α ratio in the high dose CGA and SHL group after 7 and 14 day injections, which suggested that the balance between IL-10 and TNF-α tipped towards inflammation. The ratio between IL-10 and TNF-α has been used as an accurate estimate of the inflammatory activity in the systemic circulation (27). IL-10 demonstrates potent anti-inflammatory properties through inhibiting the production of TNF-α and other pro-inflammatory cytokines (28). It has revealed to possess antioxidant-like properties in situations where oxidative stress is increased (29). On the other hand, TNF-α enhances oxidative stress both by increasing ROS generation as well as

Figure 6. The protein expression of Nox4, p22phox and p47phox in the brain tissues of rats. (A and E) Representative western blots of Nox4, p22phox and p47phox at day 7 and 14. The protein expression of (B and F) Nox4, (C and G) p22phox, and (D and H) p47phox at day 7 and 14. Lower columns: quantification of the western blotting results shown in (A and E). Data are expressed as means ± SEM of three animals. *P<0.05 vs. control group (day 7). L-CGA, low-dose CGA; L-SHL, low-dose SHL; H-CGA, high-dose CGA; H-SHL, high-dose SHL.
by decreasing antioxidants. Moreover, it has been postulated that the balance between pro- and anti-inflammatory cytokines would be related with NF-κB mediation (30). In this experiment, we also measured the protein expression of this factor and found that NF-κB p65 protein expression was increased in the lung and brain tissues when exposed to high-dose CGA or SHL injection (further study is in progress). NF-κB is one of the most important regulators of pro-inflammatory cytokines inclusive of TNF-α, IL-1β, IL-6, IL-8, and platelet activation factor gene transcription (31,32). IL-10 acts as a natural antagonist to TNF-α, by inhibiting NF-κB signaling through the preservation of inhibitory factor κB (IκB) (30). Additionally, IL-10 alleviated inflammatory stimulus mediated increase in ROS (33) and ROS mediated IκB degradation and thus activation of NF-κB (29). In general, cytokines are interwoven and regulated through a feedback mechanism (34), which suggests that other inflammatory cytokines or mediators may be present in high-dose CGA and SHL induced oxidant stress or inflammatory injury.

In summary, the present study documented the potential role of CGA in SHL injection overdose induced generating excessive peroxide in vascular endothelial cells may well be through nicotinamide adenine dinucleotide phosphate oxidase and imbalance of basal levels of pro-(TNF-α) and anti-(IL-10) inflammatory cytokines. These results illuminate mechanisms underlying the unfavorable effects of CGA induced by irrational use of Chinese herbal injection, and carefully reckon with the clinician particularly as an injection of TCM and imbalance of basal levels of pro-(TNF-α) and IL-10 at high dose, which can be partly attributed to CGA.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WYD was responsible for the production of animal models, revising and writing of the manuscript. YX contributed in the calculation of the indexes. JJY, ZH and YBZ were responsible for the collection of the data, the modification of the pictures and a part of the statistical analysis. YZ was responsible for revising and finalizing this report. All the authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the TCM Hospital of Shijiazhuang Affiliated to Hebei University of Chinese Medicine (Shijiazhuang, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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