Extraction and identification of collagen-derived peptides with hematopoietic activity from Colla Corii Asini

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Ethnopharmacological relevance: Colla Corii Asini is a widely used traditional Chinese medicine to treat anemia with a long history due to its stimulating effect in hematopoiesis, but the components contributing to this effect are still unknown. In this study, we aimed to establish a methodology to isolate the bioactive components and provide pharmacological basis for its usage in treating anemia.

Methods: 5-FU and γ-ray radiation induced anemic mice models were generated by treating with 5-FU at 150 mg/kg body weight and γ-rays by a 4 MV linear accelerator by total body irradiation using female ICR mice respectively. Oral administration of fraction A was performed by gastric lavage at 1 g/kg and 2 g/kg body weight for 12 days and 25 days and peripheral blood sample was collected from ocular sinus red blood cell (RBC) and white blood cell (WBC) counts every 3 days and 5 days for 5-FU and radiation induced models, respectively. Next, fraction A was separated to A1 and A2 using cation exchange chromatography (IEC) based on ionic strength. Fraction A1 was further separated using reverse phase chromatography (RPC) based on the hydrophobicity first with 0–10% linear gradient, then 20%, 30%, 50% constant gradient of 60% acetonitrile in neutral Na2HPO4 buffer. Peak fractions were pooled, evaporatively dried, and dissolved in ultrapure water. Finally, fraction A11 was analyzed combining tandem mass spectrometry and proteomic tools and two peptides (peptide 11 and 16) were identified. The hematopoietic effects of multiple fractions and the two peptides were measured using colony-forming units-erythroid (CFU-E), an indication of late erythroid progenitor cells and colony-forming units granulocyte-monocyte (CFU-GM), an indication of granulocyte and monocyte progenitor cells respectively on hematopoietic progenitor cells prepared from bone marrow (Till and Mcculloch 1961).

Results: Fraction A at 1 g/kg and 2 g/kg could increase RBC and WBC counts in 5-FU and radiation induced anemic mice models. Fraction A1 at 0.1 mg/ml and 0.5 mg/ml, exhibited stronger hematopoietic activity than fraction A2, both of which were subfractions from fraction A using IEX, by elevated CFU-E and CFU-GM of mouse bone marrow cells. Furthermore, fraction A11 at 0.1 mg/ml showed stronger CFU-E and CFU-GM than fractions A12 to A14 from RPC separation. Finally, peptide 11 and peptide 16 were identified from tandem mass spectrometry and peptide 11 increased CFU-E and CFU-GM in a dose dependent manner.

Conclusions: We combined multiple approaches including chromatography, mass spectrometry, cell-based assays, as well as animal studies to identify and demonstrate that the hematopoietic effect of Colla Corii Asini is at least in part from the peptidic components identified using our methodology. This is the first time to isolate peptidic components from Colla Corii Asini, and to provide molecular basis for its usage in treating anemia, which may particularly have the potential to benefit cancer patients suffering from myelosuppression due to radiotherapy or chemotherapy.

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1. Introduction

Colla corii asini (Donkey-hide gelatin, E-Jiao) is one of the well-known traditional Chinese medicines (TCMs) and nutritional
supplements for over two thousand years (Zhonghua Renmin Gongheguo wei sheng bu yao dian wei yuan hui, 1997). It is a solid glue prepared from the skin of Equus asinus by decocction and concentration, and mainly contains amino acids, microelements, and small molecular weight collagen hydrolysate (Zhonghua Renmin Gongheguo wei sheng bu yao dian wei yuan hui, 1997). A recent review reported that about 58 compounds or chemical constituents were isolated from colla corii asini over the past few decades, including amino acids, proteins/gelatins, polysaccharides, volatile substances, and inorganic substances (Wang et al., 2014). Some pharmacological properties of colla corii asini include sedation, anticoagulation, vasodilatation, hematopoiesis, as well as enhancement of cellular immunity and radio-protection. Consequently, it has been widely used to treat gynecologic diseases (i.e., dysmenorrhea, menoxienia, metrorrhagia, abortion) and chronic diseases (i.e., anxiety, insomnia, apostaxis, hemoptysis, hematuria, hemafecia) (Li and Luo, 2003; Wang et al., 2012).

However, the knowledge about substances responsible for the hematopoietic activities of colla corii asini, and the relationship between its chemical constituents and bioactivities are still very limited. Additionally, solid colla corii asini is very inconvenient for patients to take. Therefore, the discovery of its new bioactivities, the relationship between chemical constituents and bioactivities, and improvement of oral administration are important directions to fully understand the mechanism and develop new formulations of this potentially useful medicine.

Our previous studies developed a two-phased digestion model by mimicking human gastrointestinal digestion and separated colla corii asini to three major fractions: A (MW ≤5000 Da), B (5000 Da < MW ≤8000 Da), and C (MW > 8000 Da) by ultrafiltration from the digestion fluid of colla corii asini (Wu et al., 2007). We further showed that fraction A had greater therapeutic effect than other fractions to stimulate the proliferation of the CD34+ cells and the release of EPO and GM-CSF using in vitro and in vivo methods (Wu et al., 2007). Though the amino acids in fraction A have been measured, the potential substance responsible for hematopoietic activity is not yet known.

In this study, we combined the chromatography separation and hematopoietic-activity evaluation strategies to further explore the substances in fraction A that may induce hematopoiesis-stimulating activity. We further used proteomics tools to identify two peptides exhibiting hematopoietic activity, which is the first evidence to show how peptides from colla corii asini play roles in hematopoiesis. This study provides the first evidence regarding the correlation between chemical substance and pharmacological function of the colla corii asini. Furthermore, it provides a reference methodology for studying the structure and activity relationship (SAR) of protein-derived functional substances.

2. Materials and methods

2.1. Materials

Lyophilized fraction A was extracted from the digested colla corii asini by ultrafiltration as previously described (Wu et al., 2007). IL-3, IL-6, EPO, GM-CSF, SCF were purchased from PEPRO TECH, Inc, USA. 5-fluorouracil (5-FU) was purchased from Sigma. Chromatographic system (ACTA Explorer), cation exchange matrix (High Q), reverse phase matrix (Source-RPC30), and the columns (100 mm × 10 mm, for ion exchange and reverse phase exchange) were all from Pharmacia incorporation.

2.2. Anemic animal models

Female ICR mice (Institute of Experimental Animal, Chinese Academy of Science, Shanghai, China) 5–6 weeks old were used in the study. The mice were maintained under standard conditions and had free access to untreated tap water and mouse feedstuff (Institute of Experimental Animal, Chinese Academy of Science, Shanghai, China). Each mouse was used and treated in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals.

2.2.1. Chemical-induced anemia mouse model

5-FU (Sigma) at 150 mg/kg was administered intravenously to the mice (Rich, 1991; Weiterova et al., 2000). Fraction A dissolved in saline was administered orally for 12 days from the day of 5-FU injection. The same amount of saline was administered to the control mice. Oral administration was performed by gastric lavage. Peripheral blood was collected from the ocular sinus using a heparinized capillary tube every 3 days to count red blood cell (RBC) and white blood cell (WBC). Blood collection was performed on half of the mice and on alternate eyes so that each mouse was collected every 6 days, and only half of them were punctured on the same eye twice with a 12-day interval.

2.2.2. Irradiation induced anemia mouse model

Irradiation with γ-rays was carried out by a 4 MV linear accelerator (80 cm FSD, 20 × 20 field size, 200 cGy/min dose rate) to total body irradiation (Kvacheva, 2002). Before irradiation, 10 mice of each group were put together in a round plastic box (20 cm in diameter) and the radiation exposure was set so that each mouse received the same amount of total body irradiated dose. Fraction A dissolved in saline was administered orally for 25 days from the day of irradiation. The same amount of saline was administered to the control mice. Oral administration was performed via gastric lavage. Peripheral blood was collected from the ocular sinus using a heparinized capillary tube every 5 d for 25 d. Collection was performed on alternate eyes, so that so that each mouse was collected every 5 days, and three collections were performed on each eye with a 10-day interval. The RBC and WBC counts were measured by a cell counter (ABBOTT CD3700, USA).

2.3. Hematopoietic progenitor cell colony-forming assay

Cell suspensions prepared from bone marrow and spleen were used to measure colony-forming units-erythroid (CFU-E) which indicated late erythroid progenitor cells and colony-forming units granulocyte-monoocyte (CFU-GM), which indicated granulocyte and monocyte progenitor cells, respectively (Till and McCulloch, 1961). All cells were cultured in Iscove’s Modified Dulbecco’s Medium (IMDM) containing 30% fetal bovine serum, 1% bovine serum albumin, 0.8% methyl cellulose, 0.1 mM 2-mercaptoethanol, 2 U/ml mEPO (for CFU-E) or 20 ng/ml GM-CSF, 10 ng/ml IL-3, plus 10 ng/ml IL-6 (for CFU-GM). Five replicates of bone marrow cells and spleen cells (4 × 104 cells/ml for CFU-E, 2 × 103 cells/ml for CFU-GM,) were incubated in a humidified incubator at 37 °C containing 5% CO2. The colonies were counted at day 3 for CFU-E and at day 7 for CFU-GM using an inverted microscope.

2.4. Chromatographic separations

2.4.1. Ion exchange chromatography (IEC)

IEC was applied to separate the fraction based on ionic strength, particularly to remove small molecules in the current study. AKTA-explorer 100 (GE healthcare, USA) was equipped with an ion exchange column (100 mm × 10 mm, Pharmacia, Sweden) filled with High-Q anion exchange matrix (Pharmacia, Sweden). The elution was carried out with 50% constant gradient of 1 m NaCl, 50 mM Tris–HCl pH 7.4 buffer at 3 ml/min flow rate at 25 °C. Absorbance were monitored at 214 and 280 nm. Peak fractions and
flow-through were pooled, evaporatively dried, and dissolved in ultrapure water for further analysis.

2.4.2. Reverse phase chromatography (RPC)

RPC was applied to separate the fraction based on the hydrophobicity. AKTA-explorer 100 (GE healthcare, USA) was equipped with a reverse phase column (100 mm × 10 mm, Pharmacia, Sweden) filled with RESsource-RPC30 matrix (Pharmacia, Sweden). The elution was carried out first with 0–10% linear gradient, then 20%, 30%, 50% constant gradient of 60% acetonitrile in 10 mM Na2HPO4 buffer at pH 7.0 for 3 min each at a flow rate of 3 ml/min at 25 °C. Absorbance was monitored at 214 and 280 nm. Peak fractions were pooled, evaporatively dried, and dissolved in ultrapure water.

2.5. Mass spectrometry (MS)

MS was performed as described previously (Gomes et al., 2005). Basically, a Finnigan linear ion trap-Orbitrap (LTQ-Orbitrap) mass spectrometer (Thermo Electron, San Jose) with dynamic online nano-Electrospray ion source (Thermo Electron, San Jose) was used. The mass spectrometer was operated in data-dependent mode to switch between survey scan MS mode and tandem MS scan mode. The survey scan MS was acquired at the Orbitrap sector between m/z 400 and m/z 2000 with the resolution of 60,000 at m/z 400. Three most intense ions were selected for tandem MS scan, which was acquired at the linear ion trap sector where the ions were fragmented using collision-induced dissociation. The AGC target values were 3 × 105 for MS scan and 1 × 105 for tandem MS scan, respectively. The general mass spectrometric conditions were: spray voltage, 1.8 kV; no sheath and auxiliary gas flow; ion transfer tube temperature, 200 °C; normalized collision energy, 30% for tandem MS scan. Tandem MS scan ion selection threshold was 1000 counts, with the activation q as 0.25 and activation time of 30 ms. Ions with charge number 1 was excluded for tandem MS scan and a preset list was used to exclude known contaminants. Ions already used for tandem MS scan were dynamically excluded for 30 s.

2.6. Protein identification

Protein identification was performed as described previously (Duan et al., 2006). Peptides and proteins were identified via database searching by TurboSequest algorithm version 2.7 (Thermo Electron, San Jose, under license from University of Washington) of tandem MS scan spectra against NCBI Refseq human protein sequence database (ftp://ftp.ncbi.nih.gov/refseq/H_sapiens/H_sapiens/protein) containing 27,960 entries. The general database searching conditions were as follows: precursor mass tolerance as 25 ppm, trypsin as specified enzyme, MS tolerance as 1.0 Da, full tryptic specificity allowing for up to two missed cleavages, and preset cleavable ICAT as static and differential modification. The filters for the identified proteins were: Xcorr > 1.9, 2.7, 3.5 for charge number 1, 2 and 3+ respectively; deltaCn > 0.1.

3. Results

3.1. Confirm the hematopoietic effect of fraction A in vivo

In order to evaluate the hematopoietic effect of fraction A in vivo, the 5-FU induced anemic model and γ-ray irradiation induced anemic model were established. In the 5-FU induced anemic model, a single injection of 5-FU (150 mg/kg body weight) resulted in a continuous decline of WBC (20%) and RBC (75%) counts from day 1 to day 6, followed by an increase afterwards in the control group (Fig. 1). Neither WBC nor RBC count of control group could reach the initial normal value (100%) within the observed time period (25 days). Oral administration of fraction A using fraction A (2 g/kg or 1 g/kg body weight) significantly and constantly ameliorated WBC and RBC counts throughout the experimental period (p < 0.05) in a dose-dependent manner. Both counts recovered to approximately 80–90% at day 12, despite an increasing trend after day 5 in all groups (Fig. 1). These results revealed the protective or therapeutic effect of fraction A on the anemia caused by 5-FU.

In the γ-ray irradiation induced anemic model, WBC and RBC counts promptly decreased to 20% or 50% respectively after 3.5 Gy γ-ray radiation, reached the nadir on day 5, and slowly recovered afterwards. Neither WBC nor RBC count of the control group could reach the initial normal value (100%) within the observed time period (25 days). Similar to results shown in the 5-FU induced model, oral administration of fraction A (2 g/kg or 1 g/kg body weight) significantly and constantly ameliorated both WBC and RBC counts (P < 0.01) in a dose-dependent manner (Fig. 2). These results indicated the protective or therapeutic effect of fraction A on the anemia caused by 3.5 Gy γ-ray irradiation.
3.2. Separate fraction A using IEC and evaluate hematopoietic effect of the fractions

Due to fraction A’s hematopoietic effect in anemic animal models, we sought to identify its functional components using ion exchange chromatography (IEC) based on ionic strength. We first tried a linear gradient elution, but yielded only one elution peak. We next used 50% constant elution and were able to separate the fraction A into fractions A1 and A2 (Fig. 3). After the dialysis and evaporation, the dried A1 and A2 were dissolved in ultrapure water to test stimulating effect on colony-forming unit of erythrocyte (CFU-E) and granulocyte-macrophage (CFU-GM), which indicate hematopoietic activity on erythroid progenitor cells and granulocyte-monocyte progenitor cells respectively (Fig. 4). The result indicated that fraction A1 (500 μg/ml, 100 μg/ml) could dose-dependently stimulate CFU-E and CFU-GM of mice bone marrow cells. The increase rates for 500 μg/ml and 100 μg/ml were 38.7% and 17.1% for CFU-E, and 27.8% and 19.7% for CFU-GM, respectively. Meanwhile, fraction A2 displayed less stimulating effect on the hematopoietic progenitor cells in mice bone marrow, with the increase rates for 500 μg/ml and 100 μg/ml of 15.7% and 12.8% for CFU-E, and of 13.1% and 8.2% for CFU-GM, respectively. Taken together, though both fraction A1 and A2 showed stimulating effect on CFU-E and CFU-GM, fraction A1 plays a major role (Fig. 4).

3.3. Separate fraction A1 using RPC and evaluate the hematopoietic effect of fractions

To further separate the components in fraction A1, reverse phase chromatography (RPC) was applied based on the hydrophobicity of the constituents (Fig. 5). In the pilot separation, we found that only neutral buffer rather than acidic or alkaline buffer could separate fraction A1 efficiently. Therefore, phosphate buffer pH 7.0 was used at different elution concentrations to separate fraction A1 into four parts: A11, A12, A13, and A14 (Fig. 5). After dialysis, the hematopoietic effect of lyophilized fractions was evaluated again using colony-forming unit assay of hematopoietic progenitor cells. When A11, A12, A13, or A14 was supplemented into the medium at the same dose (100 μg/ml), fraction A11 exhibited the highest stimulating effect on CFU-GM and CFU-E with the increased rate of 47.4% and 46.9% respectively. Though other fractions also showed stimulating effect on the CFU-E and CFU-GM, A11 was the most potent one (Fig. 6).

3.4. Identify peptide using mass spectrometry

Next, we focused on separating components of fraction A11. Our initial trial using high performance chromatography failed, because the elution curve was too complicated to perform mass spectrometry studies. Apparently, the elution peaks were close to each other with similar characteristics (data not shown). Considering this situation, we switched to the Finnigan linear ion trap-Orbitrap (LTQ-Orbitrap) mass spectrometer with dynamic on-line nano-Electrospray ion source for the tandem MS scan of all the single peaks in fraction A11. Peptide and protein were identified by analyzing tandem MS scan spectra using TurboSequest algorithm version 2.7 and searching against NCBI RefSeq human protein sequence database which contains 27,960 entries. Combining MS and proteomics tools, two peptides were identified as peptide 16 (APGAPGPVPGPAK) and peptide 11 (VPGPMGPGSR), both derived from the protein subunit of bovine collagen alpha-1 (I) chain (Fig. 7). The match scores for both peptides were higher than 2.0 (Fig. 7, table), indicating the reliability of protein target corresponding to the peptides.

3.5. Evaluate stimulating effect of peptides on CFU-E and CFU-GM

In order to confirm the stimulating effects of the peptides on CFU-E and CFU-GM, we synthesized and tested the peptides using.
the colony-forming unit assay on hematopoietic progenitor cells. The results showed that peptide 11 could stimulate the colony formation of hematopoietic progenitor cells in a dose-dependent manner ranging from 0 to 400 μg/ml. The stimulating rates for CFU-E and CFU-GM reached 21.4% and 42.3% respectively at 100 μg/ml. As to peptide 16, the stimulating effects on CFU-E and CFU-GM were relatively low (Fig. 8).

4. Discussion

*Colla corii asini* is one of the most famous traditional Chinese medicines widely used in clinical treatment for anemia with a long history. Though many clinical trials and anemic animal models have proved its efficacy on the proliferation of erythrocyte or granulocyte precursor cells, the main substances responsible for its stimulating effect are still unknown. In this study, we applied multiple separation strategies to identify functional components in *colla corii asini* that were responsible for its hematopoietic stimulating effect. We started from fraction A to A1, A11, and finally to two peptides, which were further confirmed as from bovine collagen alpha-1 (I) chain and showed the stimulating effect in a dose-dependent manner using anemic models induced by 5-Fluorouracil or γ-ray, and colony-forming unit assays measuring CFU-GM and CFU-E.

*Colla corii asini*’s nutritional roles or therapeutic effects are mainly attributed to stimulating hematopoiesis. This is quite important especially for patients receiving intensive radiotherapy and chemotherapy to treat advanced malignant tumors and neoplastic hematologic disorders. The clinical relevance of stimulating hematopoiesis is the fact that myelosuppression is a major side-effect which limits the use of radiotherapy or chemotherapy (Chen et al., 2007). Hematopoietic and immune reconstitution can be achieved by stimulating the proliferation and differentiation of preserved hematopoietic stem and progenitor cells (HSPC) using cytokines as a protective substitution in the neutropenic period after radiotherapy, as shown in a study of co-administration of cytokines to
treat radiation-induced myelosuppression (Frasca et al., 2000). However, repeated and prolonged co-administration of cytokines to radiotherapy in patients has been reported to induce adverse effects such as pro-inflammatory and immunogenic activity (Li et al., 2001; Karlin et al., 2005). Fufang ejiao jiang (a formulation of colla corii asini) was clearly confirmed to promote the recovery of bone marrow hematopoietic function in myelosuppressed mouse model. Liu et al. (2014), attributed this effect to improvement of bone marrow hematopoietic microenvironment, facilitation of cell proliferation, prevention of bone marrow nucleated cells (BMNCs) from apoptosis, stimulation of the expressions of some cytokines, and inhibition of the TGF-β expression (Liu et al., 2014). Our identification of the bioactive components, particularly individual peptides further provides the pharmacological basis for its application in hematopoiesis so as to expand its benefits for cancer patients suffering from myelosuppression due to radiotherapy or chemotherapy.

Interestingly, another peptidic product called scorpion venom peptides (SVP) separated from scorpion Buthus Martti Karsch could also promote hematopoietic recovery. Dong et al. (2009) attributed this effect to elevation of the cytokine release including SCF, IL-1α, IL-6 and GM-CSF in bone marrow (BM) and in serum, as well as the proliferating index (PI), and the colony-forming unit numbers

Fig. 7. Tandem mass spectra for precursor ions. A): For precursor ion: 650.85⁺, the sequence was identified as APGAPGAPGVPAGK, corresponding to protein subunit of bovine collagen alpha-1 (I) chain; B): for precursor ion 526.27⁺, the sequence was identified as VPGPMGPSGPR, corresponding to protein subunit of bovine collagen alpha-1 (I) chain.
of bone marrow cells in sub-lethal irradiation-induced myelosuppression mice. SVP is thus considered as a substituent for hematopoietic cytokine combinations to accelerate hematopoietic recovery after irradiation (Dong et al., 2009). This comprehensive study also supports the application of using the peptide-derived component in hematopoiesis.

Therapeutic peptide is a very challenging area. Compared with plant-derived medicines, protein or peptide-derived medicines require a biological conversion process such as enzyme digestion or biodegradation, which usually becomes a limiting factor particularly for large protein-derived enthomedicines like colla corii asini, since they are difficult to be absorbed by the gastrointestinal system directly, and patient have to boil or stream it before taking to further make it easier to be digested. This is also one of the major reasons why we are trying to identify the bioactive components inside colla corii asini. Our study aimed to effectively enrich and identify the fundamental material basis underlying the hematopoietic activity of acolla corii asini, so that we can produce these constituents using a more cost-effective method and facilitate oral administration. If we could further chemically modify the peptide to improve its bioefficacy, it would even largely benefit patients.

However, though providing a feasible methodology of tracking bioactive components, we would like to emphasize that we may miss other components in colla corii asini that bear hematopoietic activity or other kinds of bioactivities due to the limitation of our methodology. We believe that with improved separation and detection technology, as well as bioassays, more bioactive components will be identified.

To summarize, our study identified two peptidic components from bovine collagen alpha-1 (I) chain in Colla Corii Asini that are mainly responsible to its hematopoietic stimulating effect. The data reported in this paper has deepened our understanding of the molecular basis of its nutritional and therapeutic roles for patients suffering from anemia, especially for cancer patients who develop the myelosuppression due to radio- or chemo- therapy. Furthermore, the complicated components in TCMs, either plant- or animal-derived, limit their acceptance by modern medicine practice due to lack of specific components identified or pharmacological studies. Our study thus provides a general scheme of how to explore functional components in TCMs using scientific approaches. Such an approach provides scientific evidence for the therapeutic potentials of traditional medicines which can lead to further recognition and incorporation of traditional medicines in health systems globally.

Fig. 8. Stimulating effect of the identified peptides on hematopoietic progenitor cells. The stimulating effect of peptide 16 and 11 on CFU-E (A) and CFU-GM (B), respectively. Synthetic peptide was added to the bone marrow cells cultured in the semi-solid cellulose medium. After culturing for 7 days, all colonies were counted under inverted microscope. *: p < 0.05, **: p < 0.01, compared to control group.

Author contributions

H.W., Y.Q., Y.Z., and J.L. conceived and designed the research; W. H. performed the experiments with the assistance from F.Y., Y.Q., Y. Z., and J.L. supervised the research; H.W. and C.R. wrote the manuscript.

Conflict of interest

Y.Q. is the president of Shandong Dongeejiao Group. All other authors declare that there are no conflicts of interest.

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