The Effects of Salt-Water Processing of *Phellodendri Chinensis* Cortex on the Enhancement of Kidney Absorption of the Main Alkaloids

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Abstract

*Phellodendri chinensis* cortex (PC) is a commonly used traditional Chinese medicine for clearing heat. It is often applied in the clinical practice by using the salt-water processing method. According to the theory of Chinese materia medica processing, after being processed with salt-water, traditional Chinese medicine can increase absorption of the kidney and enhance the effects of kidney disease treatments, which can be abbreviated as “entering into kidney by processing with salt-water”. PC is a typical traditional Chinese medicine using the salt-water processing method. The resulting salt-water processed PC (SPC) can specifically enter the kidney meridian, as compared to raw PC (RPC), which enhances the kidney yin nourishing and purges away kidney fire. However, the effect of “entering into kidney by processing with salt-water” of PC has not been proven and its mechanism is unknown. Therefore, it is of great significance to compare the renal absorption effect of PC before and after salt-water processing and elucidate its mechanism. In this article, using the PC alkaloid content as an indicator, a human proximal tubular epithelial cell (HK-2 cell) experiment and the binding rate of alkaloids to lysozyme-kidney target enzyme was conducted to simulate kidney absorption in vitro. These results were combined with an investigation of the alkaloid content in rat kidneys after a gavage of PC solution, comprehensively evaluating the difference in kidney absorption after salt-water processing. Compared with RPC, the efflux of SPC alkaloids in the HK-2 cell permeability experiment was significantly reduced, and affinity of the lysozyme-kidney target enzyme was higher. In addition, those who were given SPC had a higher alkaloid concentration in the kidneys than the RPC group. Finally, we verified enhanced kidney absorption effect of PC by salt-water processing, and to a certain extent revealed the mechanism of enhanced kidney absorption of SPC.

Keywords

*phellodendri chinensis* cortex, salt-water processing, HK-2 cell, lysozyme, renal absorption

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Introduction

Processing of Chinese Materia Medica is pharmaceutical technology adopted according to the theory of TCM (Traditional Chinese medicine), the needs of dialectical medication, the nature of the drug itself, and the different requirements of dispensing preparations. There are many effects of Chinese Materia Medica processing, one of which is altering or enhancing the acting tendency and location of the drug, which means that it can influence the channel tropism of the drug. Channel tropism refers to a drugs’ selective effort on a certain part of body. In other words, a certain medicine works or has obvious effects on some viscus or meridian, but has few effects on others. Clinically, if a drug reaches several meridians, its effects would be dispersed. Processing of Chinese Materia Medica can make drug effects focus on one certain part of the body. For example, Radix Bupleuri (Chaihu), Rhizoma Cyperi (Xiangfu) are attributed to the meridians of liver, the effects concentrate on the liver meridian and show greater effectiveness after being processed with vinegar. This experience summarized by

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ancient pharmacists is “entering into the liver by processing with vinegar”. Similarly, there are also “entering into spleen by processing with honey” and “entering into kidney by processing with salt-water”. Salt-water processing is one of the common Chinese Materia Medica processing methods. Although the theory of “salt-water processed into the kidney meridian” is clinically accepted in TCM, the mechanism is indefinite, i.e. the theory of “entering into kidney by processing with salt-water” has not been experimentally verified.²

*Phellodendri chinensis* cortex (Chinese name: Huangbo, abbreviated PC) which is dried bark of *Phellodendron chinensis* Schnider. (Fam. Rutaceae), has been applied for thousands of years in TCM clinical practice. PC has the effect of clearing heat, drying dampness, purging fire, relieving steaming, and removing toxins to treat soreness. In clinical use settings, PC can be used to treat diarrhea, dysentery, jaundice, dark urine, abnormal vaginal discharge and pudendal itching, seminal emissions, among other afflictions.³ One of the common processing methods of PC is processing with salt-water. In TCM theory, PC processed with salt-water (SPC) leads drug effects to the kidney meridian and possesses the functions to moderate the bitter flavor and dryness of the raw PC (RPC), enhances drug effects to nourish kidney yin, purge away ministerial fire, and clear away asthenic fever. However, PC’s theory of “entering into kidney by processing with salt-water” is still limited to the traditional theoretical formulation, lacking modern systematic research. In addition, the phenomenon of “entering into kidney by processing with salt-water” has neither been confirmed. In this article, we examined the uptake and handling in human renal proximal tubular HK-2 cells, the affinity rate with lysozyme of active alkaloid components between RPC and SPC in vitro, and the absorption of alkaloid components in rat kidney. The above three experiments were performed to verify the theory of “entering into kidney by processing with salt-water” of PC and partially explain the mechanism of this theory. The main active ingredients of PC are alkaloids, represented by berberine, phellodendrine, magnoflorine, palmitine, and jatrorrhizine.⁴ These alkaloids have significant anti-inflammatory, antibacterial, and antidiabetic effects.⁵ Besides alkaloids, there are limonoïd-type triterpenes, such as obacunone and limonin in PC,⁶ and they exhibit significant activity of antitumor⁷ and antibacterial,⁸ etc. In this article, the above alkaloid components will be used as indicators of the differences in the absorption of RPC and SPC in the kidney. The experimental data will be used to verify the theory of “entering into kidney by processing with salt-water” of PC.

The HK-2 cell line is an immortalized human proximal tubular epithelial cell. Under cell culture conditions, it can be grown on a porous and permeable poly membrane to achieve integration and spontaneously differentiate into epithelial-like cells, forming a continuous single layer cell. Its structure and biochemical effects are mainly characterized by the following aspects: ◎ well-developed microvilli ◎ polarization distribution of brush-like border enzymes ◎ various connections between cells, especially tight junctions, ◆ and differentiation of the two sides of the monolayer cells, which leads to microvilli appearing on the cell surface. One of the sides is similar to the mucosal side of the proximal tubule epithelium facing the lumen, also called apical (AP), and the other side is similar to the proximal tubule epithelium facing the basement membrane side of the kidney, also known as basolateral (BL). This cell model has been widely used in drug renal absorption currently.⁹,¹¹

This article also uses a targeted affinity technique in which the compound to be tested is mixed with the active protein under appropriate conditions to bind the receptor-ligand complex and the unbound compound. The unbound portion is then removed by ultrafiltration, and following this the bound portion is treated with an organic solvent to release the originally bound compound from the receptor. Finally, the dissociated small molecule is detected with a UPLC-QqQ-MS technique. This method is capable of screening compounds with the appropriate activity, and has been used as an important tool in screening drugs that bind to target proteins.¹²,¹³ Currently, the reported renal targeting enzymes lysozyme, peptidase, and cytochrome C can be used as carriers of renal targeting precursor drugs.¹⁴ In this experiment, extracts of the RPC and SPC were mixed with lysozyme to investigate the binding rate of PC alkaloid components to lysozyme, and then to investigate the differential kidney absorption of different processed PC products from the perspective of kidney targeting enzyme binding.

The third experiment of this article was carried out to analyze the differences in rats’ kidney absorption of the RPC and SPC by gavage administration of corresponding aqueous decoction. The kidney tissue samples were extracted at different time points, and the contents of the main PC alkaloids in kidney tissues were determined by a UPLC-QqQ-MS method. Finally, the concentrations of alkaloid compounds in rat kidney tissues were determined to compare the differences in the absorption in the kidney between RPC and SPC. The specific experiment process of this article is shown in Figure 1.

Materials and Methods

Materials and Animals

Lysozyme, PBS solution, reference substances of berberine, phellodendrine, magnoflorine, palmitine, jatrorrhizine, and nimodipine, DMEM/F-12 (1:1) medium, neonatal fetal bovine serum, CCK-8 kit (purchased from Dalian Meilun Biotechnology Co., Ltd), Micrococcus lysogenicus powder (purchased from Shanghai Yuanye Biotechnology Co., Ltd), Trypsin (Gibco Inc), penicillin double antibody solution (purchased from Hyclone, Co., Ltd), non-essential amino acid NEAA, HBSS solution (purchased from Solarbio), Transwell cell culture chambers 6.5 mm diameter inserts, 0.4 μm pore size purchased from Corning Co., Ltd), alkaline phosphatase kit (purchased from NanJing JianCheng Bioengineering Institute), mass spectrometry grade methanol and acetonitrile (purchased from Sigma), chromatography grade methanol, water as ultrapure water, non-iodized salt (purchased from Dalian Salt Industry Co., Ltd),...
and YM-100 ultrafiltration membranes (Millipore) were used in the study. Human proximal tubular epithelial cells (HK-2 cells) were purchased from the cell bank of the Chinese Academy of Sciences.

Preparation of RPC and SPC and Their Lyophilised Powder Configuration

The bark of *Phellodendri chinensis* cortex were collected in Ya’an City, Sichuan Province (lot: 180828) and authenticated by Prof. Feng Li; Voucher specimens have been deposited in the herbarium of Chinese Materia Medica processing engineering center of Liaoning province, Liaoning University of Traditional Chinese Medicine.

The preparation of RPC: An appropriate amount of PC was collected, purified and softened, cut into shreds, dried and collected. SPC: the shredded *Phellodendri chinensis* cortex was purified, added with 2% salt, prepared into salt water, smothered for 2 h, then heated in a pot at 150 to 160°C, stir-fried for 6 min, cooled, and collected.

RPC and SPC were taken separately and decocted twice with 10 times the amount of water for 60 minutes each time, and the filtrates from all times were combined separately. Then the extract of each concoction of RPC and SPC was concentrated, and the each concentrated concoction was freeze-dried for 18 h to obtain the corresponding PC lyophilized powder.

Proximal Renal Tubular Epithelial Cells Permeation Assay

Primary Culture of HK-2 Cells (Renal Proximal Tubular Epithelial Cells)

Proximal renal tubular epithelial cells (HK-2 cells) were cultured in cell culture flasks in a 37°C CO₂ incubator. The medium was changed every 2 days using conventional medium (DMEM/F-12 [1:1] + 10% FBS + 1% NEAA + 1% double antibodies), and cells were passaged when they were plastered to cover 75% of the bottom of the cell culture flask. Logarithmic growth phase cells were selected for a later study of the uptake of active ingredients in HK-2 cell permeation assay.

Cell Passaging and Culture of HK-2

Logarithmically grown HK-2 cells were taken out of the incubator and after removing the original culture medium, the cells were washed 2 times with PBS solution prior to addition of trypsin for digestion. After incubation for 10 min, the cells were taken, lightly blown, and observed to shrink to a round shape before adding DMEM/F-12 (1:1) medium to terminate the digestion. The cells were then transferred to a new cell culture flask using a pipette after repeated blowing of the culture medium in the cell preparation sample, and up to 5 mL of new DMEM/F-12 (1:1) medium was added. The cells were passaged approximately every 3 days.15

Effects of RPC and SPC Administered Solution on the Proliferation of HK-2 Cells

The cellular dosing concentration of PC was determined by the CCK-8 method. HK-2 cells at the logarithmic growth stage were adjusted to a cell density of 5 × 10⁶ mL⁻¹. 100 µL of cell suspension was inoculated into a 96-well plate and incubated in a CO₂ incubator for 24 h. 10 µL of the previously prepared RPC and SPC solutions at different concentrations (0.1 µg/mL, 0.2 µg/mL, 1 µg/mL, 2 µg/mL, 10 µg/mL, 20 µg/mL, 100 µg/mL, 200 µg/mL, 1000 µg/mL, 2000 µg/mL) was added to the experimental wells, and 10 µL of HBSS was added to the blank wells. 3 parallel wells were set up for each concentration. The 96-well plates were incubated for 48 h, then 10 µL of CCK-8 solvent was added and detect the optical density A value at 450 nm by enzyme-labeled instrument
after a 2 h incubation. The mean of A values of 3 parallel wells in each group was taken. The cell proliferation rate (%) was calculated by the following formula.

Cell proliferation rate = \frac{A_{\text{experimental group}}}{A_{\text{blank group}}} \times 100\%.

The experimental concentration screen with the maximum proliferation rate was subsequently used to examine the permeation assay.\(^{16}\)

**Establishment of a Renal Proximal Tubular Epithelial Cell Resorption Model**

HK-2 cells in a logarithmic growth phase were digested as described above and centrifuged. The old medium and trypsin were removed, and DMEM/F-12 culture was then re-added to prepare a cell suspension. The cell concentration was adjusted to 5 \times 10^6 mL\(^{-1}\) and 100 \(\mu\)L of the cell suspension was taken and inoculated into 24-well Transwell cell chambers and continued the incubation in a CO\(_2\) incubator.

**Measurement of Transepithelial Electrical Resistance (TEER) in HK-2 Cells**

The integrity of a monolayer is expressed in terms of the transepithelial electrical resistance of the cell. In general, monolayers are considered to be dense and intact if the TEER is greater than 200 \(\Omega\). Higher TEER values indicate denser monolayers. In the experiments, adjusted concentrations of HK-2 cells were inoculated in Transwell cell chambers and the transepithelial electrical resistance was measured daily using a cell resistance meter.

The transepithelial electrical resistance measurement system was first adjusted by soaking the electrode in 70% ethanol for more than 30 minutes and then equilibrating in serum-free medium for 10 minutes. The medium was removed from the culture dish of the cells to be tested, and fresh 37 °C medium was added prior to 30 minutes of equilibration. An electrode was inserted to measure transmembrane resistance. The cell transepithelial electrical resistance was obtained by measuring and averaging three readings, and subtracting the reading of the blank group with no cultured cells.\(^{17}\)

**Determination of HK-2 Cell Polarity via Alkaline Phosphatase kit Assay**

After inoculation of HK-2 cells in Transwell cell chambers, the cell monolayers were gently washed twice with Hanks solution (pH = 7.4) and incubated for 20 min at 37 °C at 1, 3, 5 and 7 days. The solutions inside and outside the wells were aspirated and the alkaline phosphatase activity of the AP side and BL side of the cell monolayers was determined according to the alkaline phosphatase kit method.

**Unidirectional Uptake of RPC and SPC Alkaloids in HK-2 Cells**

HK-2 cells cultured in Transwell cell chambers in a logarithmic positive growth phase were washed 3 times with 37 °C HANKS solution. For transfer from the AP to the BL side: 150 \(\mu\)L of high and low dose RPC and SPC solutions were added to the AP side as the donating chamber and the BL side as the receiving chamber, and 100 \(\mu\)L of solution was collected in the receiving chamber at 30, 60, 90, 120, 150, and 180 min. For the transfer from the BL side to the AP side: 1 mL of high and low dose RPC and SPC solutions were added to the BL side as the donating chamber and the AP side as the receiving chamber, and 100 \(\mu\)L of PC administration solution through the receiving chamber was collected at 30, 60, 90, 120, 150, and 180 min. 3 replicate wells were made in parallel and the same volume of PBS solution was added after the solution was collected in the receiving chamber during the experiment.\(^{18}\)

At the beginning of the experiment, 1 mL of HBSS solution was added to the BL side in the AP→BL uptake direction, while 150 \(\mu\)L of HBSS solution was added to the AP side in the BL→AP direction, and the solution was collected on the receiving chamber side after different administration times and quantified using UPLC-QqQ-MS.\(^{19}\)

**Data Analysis of Permeability Rate**

We adopted a similar approach for investigating the permeability rate of Caco-2 cells and introduced a permeability rate algorithm to analyse the uptake and efflux of PC alkaloids in HK-2 cells in both directions.\(^{20-22}\) Permeability rate in one direction of PC alkaloids in HK-2 was expressed in terms of \(P_{\text{app}}\) \(P_{\text{app}}\) was calculated from following equation.

\[
P_{\text{app}} = \frac{\Delta Q}{\Delta t} \frac{1}{AC_0}
\]

In the above formula, \(\Delta Q/\Delta t\) is the rate of the alkaloid on the accepting chamber (\(\mu\)M/s), \(A\) is the surface area of the hanging insert (cm\(^2\)) and \(C_0\) is the initial concentration of tested alkaloid in donating chamber (\(\mu\)M/mL). Based on the previous equation, we used the ratio of the permeation rate of \(P_{\text{app}}\) BL→AP to the \(P_{\text{app}}\) AP→BL as the efflux ratio to further investigate the effect of the alkaloids of PC on the uptake or efflux action of HK-2 cells.

\[
\text{Efflux ratio} = \frac{P_{\text{app}}\text{BL} \rightarrow \text{AP}}{P_{\text{app}}\text{AP} \rightarrow \text{BL}}
\]

**Assaying Differential Binding Rates of RPC and SPC Alkaloid Components to Renal Target Enzymes – Lysozyme**

**Lysozyme Activity Test**

200 \(\mu\)L of 0.1 mol/L PBS buffer (pH 7.2-8.0), 50 \(\mu\)L of PC solution, and 100 \(\mu\)L of 1000 U/mL lysozyme were added to a
96-well plate, which was mixed well and left at 4 °C for 20 min. Micrococcus lysoективus powder dissolved in 0.2 mol/L phosphate buffer (pH 6.2) was added to the plate and incubated at 37 °C. The optical density of the samples was measured at 450 nm after 20 min of reaction. A background control group and a blank control group were also set up for the experiment. The enzyme activity ratio (EAR) of lysozyme was calculated by the following formula.23

\[ \text{EAR} = \left( \frac{A_{\text{sample}} - A_{\text{background}}}{A_{\text{blank}}} \right) \times 100\% \]

In the formula, \(A_{\text{blank}}\) indicates the absorbance value of the test sample without the PC administration solution; \(A_{\text{sample}}\) indicates the absorbance value of the test sample with the addition of the PC administration solution and the enzyme; \(A_{\text{background}}\) indicates the absorbance value of the test sample without the enzyme and the PC administration solution.24

Centrifugal Ultrafiltration Assay

200 μL of 0.1 mol/L PBS buffer (pH 8.0), 10 mg/mL of RPC and SPC administration solution (high and low doses tested separately, 100 μL for the high dose and 50 μL for the low dose), and 100 μL of lysozyme enzyme solution at different enzyme concentrations (1,000 U/mL) were added to a 24-well plate and placed in a 37 °C incubator for 30 min, then transferred to centrifugal ultrafiltration tubes (YM-100). The samples were centrifuged at 12,000 r/min for 20 min, which was repeated 3 times with PBS buffer, 500 μL each time. The centrifugation solution was discarded and 120 μL of water/methanol (10:90, V/V) solution was added to the ultrafiltration tube. The tube was stabilized at room temperature for 10 min and centrifuged at 12,000 r/min for 10 min. This operation was repeated 3 times and the centrifuge solutions were combined. The combined centrifuge solutions were freeze-dried and re-solubilized by adding 50 μL of methanol/water (50:50, V/V) solution for the LC-MS assay. A positive control and a blank group were established simultaneously.25-26

Additionally, the experiments were controlled against inactivated lysozyme. After inactivation of the lysozyme by heating to 110 °C, the experiments were carried out using the same method as above and the binding rate was calculated as following.27

\[ \text{Binding Rate} = \left( \frac{A_b - A_i}{A_i} \right) \times 100\% \]

In the above formula, \(A_b\) is the peak area of the chromatographic peaks of each of the RPC or SPC alkaloids before affinity centrifugal ultrafiltration screening; \(A_i\) is the peak area of the chromatographic peaks of each of the RPC or SPC alkaloids bound to lysozyme after affinity action; \(A_c\) is the peak area of the chromatographic peaks of each of the RPC or SPC alkaloids bound to lysozyme after lysozyme inactivation.

Differential Absorption of RPC and SPC Alkaloids in rat Kidney

Laboratory Animals

Sixty-six Sprague-Dawley (SD) male rats, weighing 180 to 200 g, 6 to 8 weeks old were purchased from Liaoning Changsheng Biotechnology Co. License No.: SCXK (Liao) 2010 to 0001. The rats were acclimatized and fed for one week prior to the experiments, and received ad libitum food and water during the pre-feeding period at room temperature (22 ± 2 °C) and humidity (60 ± 2)%.

Dosing and Kidney Sample Collection

The 66 rats were randomly divided into two groups: 30 for the RPC group, 30 for the SPC group, and 6 for the Blank group. Each group was given 10 mL kg⁻¹ of the corresponding administration decoction of RPC and SPC at 1 g/mL.28 At 0.5, 1, 3, 6, and 9 h time points, 6 animals in each group were excised and the kidney tissues were immediately removed, rinsed with saline, blotted with filter paper, and stored in self-sealing bags at −80°C.29

Treatment of Kidney Tissue Samples

The kidney tissue samples collected by the above method were weighed precisely at 200 mg, cut up, added to 2 mL of physiological saline before processing with a tissue homogeniser. After centrifugation at 4000 r/min, 100 μL of the supernatant tissue homogenate was taken and 10 μL of the nimodipine (internal standard) solution was added and vortexed for 30 s. 400 μL of acetonitrile was then added and vortexed for 180 s. After centrifugation at 12,000 r/min for 10 min, the supernatant was taken and blown dry at 37 °C under nitrogen. The residue was re-dissolved by adding 100 μL of acetonitrile/water (1:1), vortex mixing for 3 min, and centrifugation at 12,000 r/min for 5 min. 5 μL of supernatant was taken for UPLC-QqQ-MS analysis.30

UPLC-MS/MS Conditions for Determination of PC Alkaloids

The alkaloid component determination in the three above experiments described herein was all carried out by a Waters ACQUITY UPLC system and a Xevo TQ-S mass spectrometer. Moreover, the chromatographic separation was performed on a Waters UPLC BEH C18 column (100 mm × 2.1 mm, 1.7 μm) at 35 °C. The mobile phase composition was 0.1% formic acid water (A) and 0.1% formic acid acetonitrile (B). The gradient elution program is shown in Table 1, and ran at a flow rate of 0.3 mL/min. Each sample was placed in an autosampler at 4 °C.

Most of the bioactive PC compounds are alkaloids, and alkaloids have strong signal response in positive scan mode. Therefore, the positive ion mode was applied to detect the samples. To receive a richer mass spectral abundance of
precursor and product ions, the MS condition was conducted as follows: source temperature 150 °C; capillary 3000 V; cone voltages 60 V; desolvation gas (N2) flow 900 L/h; desolvation temperature 500 °C; and cone gas flow 70 L/h. The majorization of collision energy was according to the chemical standards, and using helium for collision gas of collision-induced dissociation. Quantitative analysis was executed by multiple reactions monitoring (MRM). The precursor → product ion transitions of m/z 342.17 → 192.15, m/z 342.18 → 265.16, m/z 338.15 → 323.03, m/z 352.15 → 336.20, m/z 336.07 → 320.29 and m/z 419.21 → 343.17 were employed for quantification of phellodendrine, magnoflorine, jatrorrhizine, palmatine, berberine, and nimodipine, respectively. Representative positive ion chromatograms of blank kidney sample, blank kidney sample minus, and nimodipine, respectively. Representative positive ion chromatograms of blank kidney sample, blank kidney sample minus, and nimodipine, respectively.

**Results**

**Analysis of HK-2 Cell Permeation Assay**

Effects of RPC and SPC Administration Solutions on HK-2 Cell Proliferation. The optimal concentrations of different PC con-         
centrations were screened by the CCK-8 kit method, and the prolif-         
eration rates were found to be higher for both 200 μg/mL and 100 μg/mL of concentration of RPC and SPC. Therefore, in the next step of the HK-2 uptake transport study, RPC and SPC administration solutions of 200 μg/mL and 100 μg/mL were screened for analysis, with 200 μg/mL as the high dose and 100 μg/mL as the low dose.

Examination of HK-2 Cell Monomolecular Layer Integrity. During the experiment, the transepithelial electrical resistance of the cells was recorded daily. After day 7, the transepithelial electrical resistance of HK-2 cells reached 600 Ω cm⁻², and then increased slightly, and finally the TEER value remained stable at approximately 700 Ω cm⁻². The TEER changes in HK-2 cells during the experiment are shown in Figure 2.

The alkaline phosphatase activities of the AP and BL sides of HK-2 cells in Transwell cells chamber were basically the same after 3 days of inoculation. The alkaline phosphatase activity on both the AP side and BL side of the culture medium tended to increase as the incubation time increased, with the difference between the AP side and BL side gradually increasing over time. On the other hand, the increase in alkaline phosphatase activity on the BL side was slow, but by 7 days it was only twice as high as at 3 days. The ratio of alkaline phosphatase activity between the two sides was calculated to be 0.85 ± 0.09 at day 3 and increased significantly to 4.95 ± 0.13 at day 7, where it remained stable at a high level, indicating that the alkaline phosphatase activity on the AP side was higher than that on the BL side, ie the phenomenon of polarization. At the same time, the intracellular alkaline phosphatase activity increased rapidly from 3 to 7 days and then leveled off.

The above experiments confirmed that the HK-2 cells had become an intact and dense monolayer during the experiments by using the HK-2 transmembrane resistance and the alkaline phosphatase assays, and could be used for drug transport studies.

Measurement of the Uptake and Transport of RPC and SPC Administered Solutions in HK-2 Cells. The uptake and transport of phellodendrine, magnoflorine, and berberine components of RPC and SPC through HK-2 monolayer cells from AP side to BL side and BL side to AP side at the corresponding times are shown in Table S1 and Figure 3. The results showed that HK-2 cells have different uptake capacities for different alkaloids, in the order of phellodendrine > berberine > magnoflorine. In addition, the uptake capacity of both BL→AP and AP→BL transport reached a peak at 120 min in terms of uptake direction.

Analysis of PC Alkaloids of Permeability Rate. PC alkaloids showed significant efflux for HK-2 cells, ie the permeation rate for the uptake direction BL→AP was higher than the permeation rate for the uptake direction AP→BL. The efflux ratio for each alkaloid at 120 min are shown in Table 2.

Analysis of Lysozyme Binding Rate

Lysozyme Activity Test Results. Different doses of PC administration solution were combined with different enzyme concentrations and treated with HK-2 cells. After 120 min of incubation, the activity of HK-2 was measured using the CCK-8 kit method. The results showed that the HK-2 cells had different uptake capacities for different alkaloids, in the order of phellodendrine > berberine > magnoflorine. In addition, the uptake capacity of both BL→AP and AP→BL transport reached a peak at 120 min in terms of uptake direction.

| Table 1. Gradient Elution Program of Mobile Phase. |
|--------------------------------------------------|
| Time(min) | A % (0.1% formic acid water) | B % (0.1% formic acid acetonitrile) |
|----------|-------------------------------|-----------------------------------|
| 0        | 75                            | 25                                |
| 2.5      | 75                            | 25                                |
| 4.5      | 40                            | 60                                |
| 7.5      | 0                             | 100                               |

Figure 2. TEER dynamics in HK-2 cell at different culture times.
concentrations of lysozyme and it was found that lysozyme activity was maintained above 95%, indicating that PC administration solution does not affect lysozyme activity and is appropriate for use in the following binding rate assay.

Differential Binding Rates of RPC and SPC Alkaloids Components to Lysozyme. The lysozyme activity assay showed that the PC alkaloid active ingredients had a binding rate to the kidney tissue target enzyme, lysozyme, which indicates that PC alkaloid components can interact directly with the kidney and therefore exert the efficacy of PC. A comparative study of the binding rates of the PC alkaloid components with lysozyme was carried out and the SPC alkaloid components were found to have a higher binding rate compared to the RPC components. The binding rates of each RPC and SPC alkaloid component with lysozyme are shown in Table 3.

Comparative Analysis of RPC and SPC Absorption in rat Kidney

The concentrations of alkaloid compounds in rat kidney tissues were determined by UPLC-QqQ-MS at different times after gavage administration of aqueous decoction of RPC and SPC. The results are shown in Table S2 and trends are shown in Figure 4.

Sterisk denotes statistically significant differences compared with the RPC group at the same time after oral administration (* P < .05; ** P < .01)

As shown in Figure 4, the concentration of most of the PC alkaloid components decreased gradually after gavage administration in rats, while after oral administration, the alkaloid concentrations of SPC relative to RPC were generally higher in the kidney tissues of rats after the same administration time for the same tested components, indicating a trend of enhanced absorption of each component in the kidney tissues after salt-water processing.

Validation of UPLC-QqQ-MS Conditions for Quantification

The UPLC-QqQ-MS method was validated in terms of linearity, precision, repeatability, stability, and recovery. Quantification of the HK-2 cell permeability assay was performed by the standard curve method of the external standard method, while the quantification of alkaloid concentrations in rat kidney was performed by the weighted least square linear regression of the internal standard method. The $r^2$ values of the five alkaloid standards were more than 0.99, indicating good linearity (see Table S3). The intra-day and inter-day precision of the proposed methods was less than 11.25% and 9.64%, respectively (see Table S4). The Matrix effects and extraction recoveries of the method ranged from 86.24% to 108.21% and 86.31% to 98.36% (see Table S5). The stability of the method of storage at room temperature for 6 h, storage at $-80 \, ^\circ\text{C}$ for 30 days and the freeze-thaw cycles were less than 13.85% alteration of the relative intensity of each analyte (see Table S6). The above results indicated that the method was sensitive, precise, and accurate enough for quantitative analysis.

Discussion

HK-2 Cell Permeation Assay

The HK-2 cell line is a human-derived renal proximal tubular epithelial cell that grows in porous permeable Transwell cell
chambers under conventional cell culture conditions and, when cell resistance reaches a certain value, becomes a tight cell membrane that is structurally and biochemically similar to human renal proximal tubular epithelial cells with microvilli and marginal enzymes. HK-2 cells are tightly connected and can differentiate into mucosal side-like (AP side) and basolateral side-like (BL side) types, and have associated drug-carrying enzymes. By studying the resolution of natural compounds through HK-2 cells, this can be used to predict the uptake of compounds in renal tissue.

In this article, three experiments were designed to verify the effects of SPC on enhancing the kidney uptake of its alkaloid components. In the original plan, the five representative PC alkaloid components were determined in all three experiments, but in the HK-2 cell uptake assay, the responses of palmatine and jatrorrhizine were too low to be accurately quantified, so these two indicator components were omitted from the HK-2 experimental procedures.

From the result of Table 2 it can be seen that when the ratio is greater than 1, the state phenomenon of passage through HK-2 cells is considered to be efflux, while less than 1 is absorption. When the ratio of the passage rate of other alkaloid components of the same dose was compared, it was found that for the alkaloid components of PC, with the exception of magnoflorine, the efflux alkaloid components of RPC was more obvious than that of SPC at the same dose in the model of HK-2 cell. In other words, the efflux of alkaloid components of SPC was relatively weaker than RPC, indicating renal absorption of the alkaloid components enhanced after salt-water processing of PC.

Through the ability of PC on the uptake of HK-2 cells in different directions, it was found that the phenomenon of “entering into kidney by processing with salt-water” of PC was mainly due to weakened renal excretion of the alkaloids after salt-water processing of PC, so that they can be better absorbed by the kidney and achieve the effect of “entering into kidney by processing with salt-water”.

### Table 2. The Efflux Ratio of Each Alkaloid Analytes at 120 min in the Model of HK-2 Cell (%).

| Analytes    | HD of SPC | LD of SPC | HD of RPC | LD of RPC | HD of SPC | LD of SPC | HD of RPC | LD of RPC |
|-------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| phellodendrine | 0.99      | 0.76      | 1.03      | 1.14      | 0.15      | 0.55      | 0.15      | 0.33      |
| magnoflorine  | 0.15      | 0.55      | 0.15      | 0.33      | 1.13      | 2.05      | 1.63      | 3.16      |
| berberine     |           |           |           |           |           |           |           |           |

### Table 3. Binding Rates of Alkaloids with Lysozyme from RPC and SPC (%).

| Analytes    | High dose group | Low dose group |
|-------------|-----------------|---------------|
|             | RPC             | SPC           | RPC         | SPC         |
| phellodendrine | 19.05           | 22.22         | 2.41        | 13.68       |
| magnoflorine  | 7.86            | 15.93         | 8.54        | 15.27       |
| jatrorrhizine | 4.67            | 13.52         | 5.54        | 11.76       |
| palmatine     | 8.16            | 12.33         | 3.49        | 7.04        |
| berberine     | 7.63            | 21.95         | 3.63        | 18.14       |

### Figure 4. Concentrations of each analyte in rat kidney tissue after oral administration of RPC and SPC.

### Binding Rates of Alkaloid Components to Lysozyme

In addition to the alkaloid components, this article also compared the binding of limonins components in PC with lysozyme. It was found that the peak of ion pairs of limonins components were not of high corresponding intensity in UPLC-QqQ-MS analysis after the same sample treatment, suggesting that the binding rate of PC limonins components to lysozyme, the target enzyme of kidney tissue, was low, which also indicates that the PC alkaloid components are the main kidney absorption components.

Moreover, the difference in the binding rates (high and low concentrations) of each alkaloid component of PC with lysozyme reveals that the binding rates of phellodendrine and berberine components of PC are higher than those of magnoflorine, jatrorrhizine, and palmatine, indicating that there is also a selective effect of PC on alkaloid components by renal absorption.

### Absorption of Alkaloids in rat Kidney

As one of the representative traditional Chinese medicine salt-water processing treatments, PC can lead the drug into the kidney after salt-water processing, which enhances the kidney yin nourishing effects, purges away ministerial fire, and clears away asthenic fever. The results showed that the alkaloids of phellodendrine, magnoflorine, berberine, palmatine, and...
jatrorrhizine from PC were mostly higher in the salt-water processed product than in the raw product after the same administration time, thus indicating that PC alkaloids tend to be more distributed in the kidney tissue after salt-water processing, and thus treat kidney-related diseases. In the previous experiment, we replicated a rat model of kidney yin deficiency and investigated the therapeutic effects of different processed PC concoction products in rats with kidney yin deficiency, and found that PC had a better effect of nourishing kidney yin and purging away ministerial fire after salt-water processing. In this experiment, the pharmacodynamics of PC were mechanistically verified, that is, the absorption of its active ingredients in kidney tissues could be enhanced after salt-water processing.

Conclusions

In the present experiment, we determined alkaloid concentrations in the rat kidney by oral administration to confirm the effect of enhanced kidney absorption of SPC, while the efflux of alkaloids of SPC was found to be weakened relative to RPC in HK-2 cell model experiments, as well as in lysozyme affinity experiments, which revealed a higher binding rate of alkaloids of SPC to the renal target enzyme-lysozyme, thus explaining mechanistically the effect of enhanced kidney absorption of SPC. The above confirmed and explained the pharmacological theory of PC “entering into kidney by processing with salt-water”.

Data Availability

The data used to support the findings of this study are included within the supplementary information file(s).

Supplementary Information files: Table S1. Results of the concentrations of alkaloids of RPC and SPC both at the AP and BL sides after uptake through HK-2 cells at different times (ng/mL, x ± s, n = 3) Table S2. Concentrations of each analyte in rats kidney after oral administration of RPC and SPC. Table S3 to 1. The regression equations and linear ranges for the determination analytes in HK-2 cell transwell chamber solution. Table S3 to 2. The regression equations and linear ranges for the determination analytes in rats kidney. Table S4 to 1. Precision and accuracy for the analytes in HK-2 cell transwell chamber solution (n = 6). Table S4 to 2. Precision and accuracy for the analytes in rats kidney (n = 6). - Matrix effects and extraction recovery for the analytes in HK-2 cell transwell chamber solution (n = 6). Table S5 to 2. Matrix effects and extraction recovery for the analytes in rats kidney (n = 6). Table S6 to 1. Stability of each analyte in HK-2 cell transwell chamber solution under different storage conditions (n = 6). Table S6 to 2. Stability of each analyte in rats kidney under different storage conditions (n = 6). Figure S1 Typical multiple reaction monitoring chromatogram of (1) phellodendrine, (2) magnoflorine, (3) jatrorrhizine, (4) palmatine, (5) berberine and (6) IS. Blank kidney samples from six rats (a); spiked blank kidney samples with the analytes and IS (b); kidney samples from rats 1 h after oral administration of SPC extract (c).

Author Contributions

Conceived and designed the study: Fan Zhang, Tianzhu Jia. Assay of HK-2 permeation assay and drug concentration in rats’ kidney: Fan Zhang, Li Li. Assay of binding rate with lysozyme: Fan Zhang, Jiahu Zhao. Sample processing of PC: Fan Zhang, Xiutong Ge, Hui Gao. Wrote or contributed to revising the manuscript: Fan Zhang. All authors have read and approved this version of the manuscript.

Declaration of Conflicting Interests

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Ethical Approval

All experimental procedures were conducted in accordance with the guidelines approved by Animal Ethics Committee of The Affiliated Hospital of Liaoning University of Traditional Chinese Medicine (Permit Number:2019YS\[DW]-029-01).

Supplemental Material

Supplemental material for this article is available online.

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