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Original article

The comparative study on the absorption and excretion of caffeine in rats orally administered ripened and green Pu-erh tea

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Summary. *Purpose:* Ripened pu-erh tea is one of the most famous Chinese dark teas in Eastern Asian and Western China. When rats were orally administered ripened pu-erh aqueous extract (caffeine dose 135 mg/kg), the maximum plasma concentration (C_{max}) of caffeine was 53.96±9.46 μg/mL, which was significantly less than those of green pu-erh tea (90.95±20.93 μg/mL). Furthermore, area under the plasma concentration–time curve (AUC_{0-t}) of caffeine of ripened pu-erh tea was also significantly decreased. The total fecal content of caffeine of rats given ripened pu-erh tea was 4.83 mg, which was almost two-folds of the fecal caffeine of rats given green pu-erh tea (2.46 mg). The *in vitro* human intestinal epithelial cell linetransportation results also showed that the caffeine of ripened pu-erh tea was less transported from apical (AP) to the basolateral (BL) side. These results indicated post-fermented pu-erh tea impeded the absorption of caffeine but promoted its excretion.

Key words: caffeine, absorption, pu-erh tea, rats, CaCo-2

Introduction

Pu-erh teas are usually classified into ripened pu-erh tea and green pu-erh tea. Ripened pu-erh tea is post-fermented tea with mature leaves of *Camellia sinensis* var. *assamica* by fungi, while green pu-erh tea is very similar to green tea, which is unfermented or lightly fermented tea (1). Recently, the healthcare effects of ripened pu-erh tea were studied by various disease models (2,3). Pu-erh tea has been considered as an alternative plant supplementary for preventing metabolic syndromes (4). It was reported that these two types of pu-erh tea both showed significant lipids-lowering effects on hyperlipidemia rats induced by high fat diet (5). Both two kinds of teas contained high content of caffeine, the level of which was not affected by post-fermentation (6).

It has been widely reported that caffeine ingestion significantly improved performance during short-term, high-intensity exercises, sprints, and strength and power exercises (7). Additionally, caffeine affects the central nervous system (CNS) by acting as a potent adenosine receptor antagonist. As we all know, caffeine is main active compound which leads to presenting symptoms of agitation and hyposomnia and consequent complication (8).

Ripened pu-erh tea possesses a special feature, which is trusted by many consumers. It is always believed that drinking ripened pu-erh tea doesn't cause hyposomnia. Although this assertion was consumers' subjective feelings, this hypothesis and mechanism should be worthy of confirmation. This study aimed at exploring the absorption and excretion of caffeine in two types of pu-erh teas, i.e. ripened tea and green tea.

In this study, our primary objective was to compare the absorption of caffeine in ripened and green pu-erh tea using in *vitro* caco-2 model and in *vivo* rats' pharmacokinetics study.

Materials and methods

Chemicals and reagents

Ripened pu-erh tea and green pu-erh tea were purchased from Dayi Pu-erh tea Company. The contents of caffeine of ripened and green pu-erh tea were determined as 1.70% by HPLC. Methanol (HPLC grade) was obtained from TEDIA Company.

Tea extract preparation

150 g of ripened or green pu-erh tea powder was boiled using 1500 mL deionized water for 15 min, and subsequently filtered to obtain tea aqueous extract. The extract was then evaporated to dryness under reduced pressure condition. Before administered to rats, the tea extract was dissolved in water at the concentration of 100 mg/mL.

To prepare the tea extract for caco-2 transportation experiment, the tea extract was prepared by boiling 2 g of the ripened or green pu-erh tea using 100 mL of distilled water for 30 min. The extract was determined for the content of caffeine. The pH value of tea extract was adjusted to 7.0. The tea extract was then diluted with HBSS to tea solution for transportation experiment.

Trans-enterocyte transport in caco-2 cell monolayer

Caco-2 cells (passage 50~80, American Type Culture Collection, Rockville, MD, USA) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 1% nonessential amino acid solution, 100 units/mL penicillin, and 0.1 mg/mL streptomycin at 37 in an atmosphere of 5% CO_2. The cells were subcultured when they reached 80% confluency. For the in *vitro* transport study, cells were seeded on the apical (AP) compartment of transwell plates (6 well plates, 0.4 μm pore size and 4.7 cm^2 surface area, Corning Inc, Corning, MA, USA) at a concentration of 4×10^5 cells/mL (1.5 mL/well). The complete culture medium was added into basolateral (BL) compartment (3 mL). The apical and basolateral medium were replaced every other day. Cells were incubated for 18–21 days until the transepithelial electrical resistance (TEER), measured using an epithelial voltammeter (Millicell® ERS-2, World Precision Instruments, Sarasota, FL, USA), reached 250–300 R/cm^2.

Transepithelial transport study

After the culture medium was aspirated, the cell monolayers were washed three times with blank Hanks' Balanced Salt Solution (HBSS). For transportation experiments of tea extract, the apical side of the cell monolayers received 2 mL tea extract solution (whereas the other side received 2.5 mL HBSS). Cells were incubated for at 37 , and then 1.25 mL of solution was removed from BL side, 50 μL of solution was removed from AP side at 30, 60, 90, 120, and 150 min after treatment. After sampling, 1.25 mL of HBSS was replenished back to BL side. The contents of caffeine in the samples of BL side at 30, 60, 90, 120, and 150 min were then determined.

Animals

Male Sprague–Dawley rats (290–330 g) were purchased from the Anhui Medical University Experiment Animal Center (Hefei, China). Animal welfare and experimental procedures were strictly in accordance with the Guide for the Care and Use of Laboratory Animals (US National Research Council, 1996) and the related ethics regulations of Anhui Agricultural University. All animals had free access to food and water. The food was removed 12h before collection of blood samples. During the pharmacokinetics, the rats were free access to water.

In vivo absorption study in rats

Ten male Sprague–Dawley rats were randomly divided into two groups with five rats in each group to receive various administrations. In group 1, rats were orally administered ripened pu-erh tea aqueous extract. The caculated dose of caffeine in the ripened pu-erh tea extract was 135 mg/kg body weight of rat. In group 2, rats were orally administered with green pu-erh tea aqueous extract at same dose of caffeine. The blood samples (0.3 mL) were obtained *via* oculi chorioideae vein at 5, 10, 20, 30, 45, 60, 90, 120, 240, 480,

and 720 min after administration, and then transferred into a heparinized micro-centrifuge tube according to a programmed schedule. The plasma was isolated immediately by centrifugation at 6000 rpm for 10 min and stored at –20 ˚C until analysis.

Feces sample preparation and analysis

The feces from 0-60, 60-240 and 240-720 min were collected. Total feces of five rat of each group were weighed, and then extracted with 10 times of chloroform methanol (2:1) by ultrasonic extraction for 30 min at 25˚C. 100 μL of extract was diluted with 20 times of distilled water. 5 μL of the supernatant was injected into HPLC system for analysis.

Sample preparations and HPLC analysis

An aliquot of 100 μL plasma sample was placed into a centrifuge tube. The mixture was spiked with 1000 μL chloroform and vortexed for 3min, and then chloroform extract was transferred into a clean tube. The extraction was evaporated to dryness under a nitrogen gas stream in a 35˚C water bath. The residue was reconstituted in 100 μL pure water.

A 5 μL supernatant solution was injected into the High-performance liquid chromatography (HPLC) system. HPLC separations were performed on an Elite C18 analytical column (4.6×25 mm) using a Waters 600 HPLC pump and a Waters 2487 dual wavelength absorbance detector monitoring a single wavelength at 280 nm. Elution of analyte consisted of 30% methanol (A) and 70% water (B). The column temperature was maintained at 40˚C. The flow rate was 1 mL/min and the detector operated at 280 nm. The injection volume for HPLC was 5 μL.

Data and statistical analysis

Plasma concentration versus time profile was analyzed by non-compartmental methods using WinNonlin software (Pharsight Corporation, Mountain View, CA, USA, Version 5.2).The pharmacokinetic parameters, including peak plasma concentration (C_{max}), time to reach C_{max} (Tmax), and the area under plasma concentration-time curve [AUC(0-720 min)] were obtained from analysis of experimental data. A p-value less than 0.05 is considered to be significantly different using upaired student's t-test.

Results

In vitro Caco-2 cell study

As presented in Fig. 1, during the transportation, the content of caffeine of ripened pu-erh tea in AP showed a similar content as green pu-erh tea, but the caffeine's levels of ripened pu-erh tea in BL side were significantly less than green pu-erh tea. Furthermore,

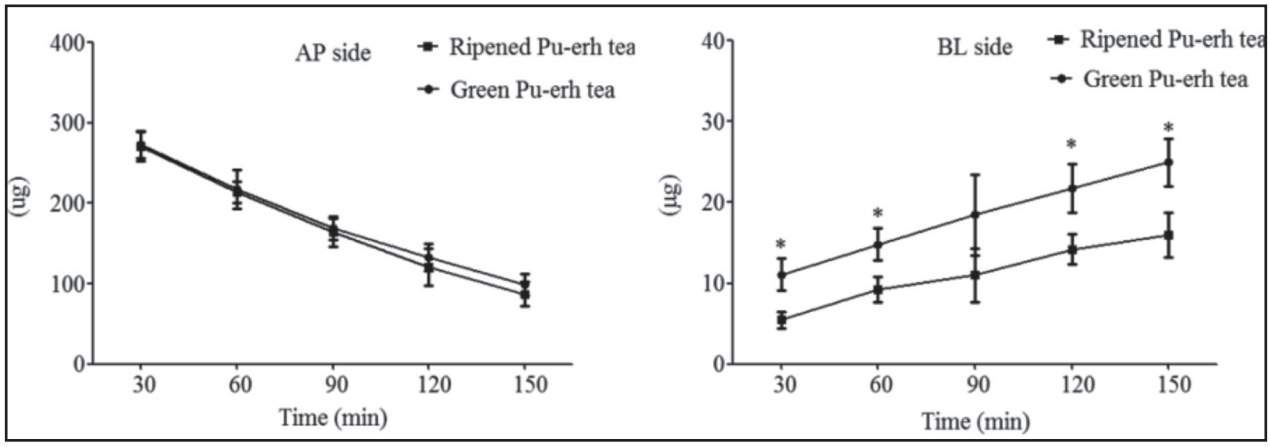

Figure 1. Time course of the permeations of caffeine from the apical to the basolateral side through caco-2 cells. Each point shows the mean±S.E.M. of three experiments. *p< 0.05 compared with green pu-erh tea.

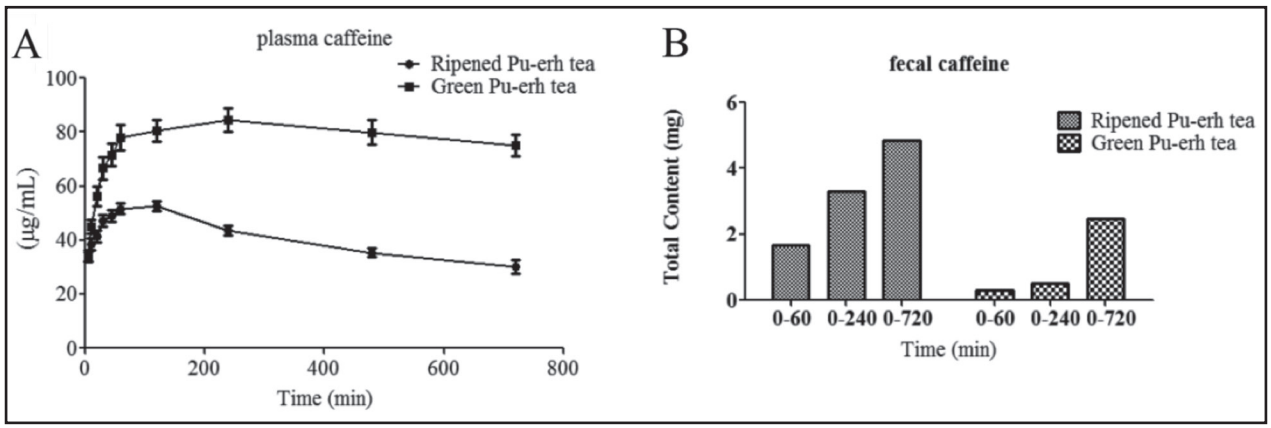

Figure 2. The time-dependent plasma levels (A)and total fecal amount of caffeine (B) in rats orally administered ripened pu-erh tea or green pu-erh tea(135 mg/kg).

the transported caffeine accounted for a small part of total caffeine of tea extract. Most of caffeine may be uptake by CaCo-2 cells. These results suggested that ripened pu-erh tea affected the transportation of caffeine.

Pharmacokinetic study

The plasma concentration versus time profile of caffeine after oral administrations of ripened pu-erh tea extract and green pu-erh tea extract was shown in Fig. 2 and pharmacokinetic parameters are shown in the Table 1.

The time to reach C_{max} (53.96±9.46 μg/mL) was 93.00±37.35 min for plasma caffeine of rats orally administered ripened pu-erh tea. But in green pu-erh tea, the time to reach C_{max} was 273.00±201.36 min. As shown in Table 1, in comparison with green pu-erh tea, the absorption of caffeine was decreased in the rats orally administered ripened pu-erh tea. For example, the C_{max} of plasma caffeine of rats orally administered ripened pu-erh tea was 53.96±9.46 μg/mL, which was significantly less than that of green pu-erh tea ($P<0.01$). Furthermore, $AUC_{(0\text{-}720\ min)}$ of plasma caffeine of rats orally administered ripened pu-erh tea was 54679.66±10407.17 μg min/mL, significantly less than that of green pu-erh tea (113116.80±2847.96 μg min/mL). These results indicated that ripened pu-erh tea decreased the oral-bioavailability of caffeine.

Table 1. Pharmacokinetic parameters for caffeine in rats after oral administration of ripened pu-erh tea and green pu-erh tea containing the caffeine dose of 135 mg/kg (mean ±SD, n =5)

Parameters	Ripened pu-erh tea	Green pu-erh tea
$AUC_{(0\text{-}720\ min)}$		
(μg min/mL)	54679.66±10407.17**	113116.80±2847.96
C_{max}(μg/mL)	53.96±9.46**	90.95±20.93
T_{max}(min)	93.00±37.35**	273.00±201.36

***p<0.01 compared with green pu-erh tea.*

After a single oral administration of ripened or green pu-erh tea extract with the caffeine dose of 135 mg/kg, the total fecal excretion of caffeine was determined and summarized in Fig.2. In the 0-720 min post-administration, the total excreted amounts of caffeine in rats orally administered ripened pu-erh tea was increased by 95.1% compared with green pu-erh tea group.

Discussion

So far, there was few reports about the pharmacokinetics study of caffeine in rats, but many human pharmacokinetics studies showed that caffeine was rapidly absorbed, but slowly eliminated (13,14). It was reported that the aborption of caffeine was different between pure caffeine and caffeine in green tea extract (15). When rats was given pure caffeine and green tea extract at the dose of 73 mg/kg, the $C_{max}$and $AUC_{(0\text{-}t)}$were 9.18±1.39/5.5±1.66 μg/mL and 63.74± 10.49/39.68±11.33 μg h/mL (15). It was apparent that

other compounds of green decreased the absorption of caffeine. It was suggested that L-theanine of green tea may affect the absorption of caffeine in *vivo* (9). In the ripened pu-erh tea, the level of L-theanine of ripened pu-erh tea was highly decreased (10). It was suggested that other unique compounds may affect the absorption of caffeine of ripened pu-erh tea. With references of many reports about tea bioavailability (11,12), the pharmacokinetics of caffeine of pu-erh tea in *vivo* was less reported, not to mention the comparative study of ripened and green pu-erh tea.

In the present study, the ingested caffeine dose was 135 mg/kg, but the $C_{max}$and $AUC_{(0-t)}$ were 53.96±9.46/90.95±20.93 μg/mL and 54679.66±10 407.17/113116.80±2847.96 μg min/mL (equal to 911.33±173.45 and 1885.28±47.47 μg h/mL) for ripened and green pu-erh tea. According to the human absorption study using different doses of caffeine, the caffeine kinetics were nonlinear (16,17). The higher dose of caffeine consumption showed better oral bioavailability (17). Compared with the previous resport (15), the higher C_{max} and $AUC_{(0-t)}$ of caffeine in the present study may be attribted to the nolinear pharmacokinetics of caffeine *in vivo*. The results suggested that the absorption of caffeine was highly affected by dosing range and other compounds in tea.

To clarify the absorption of caffeine in rats, it is necessary to analyze the pharmacokinetics parameters of caffeine between ripened and green pu-erh tea. The raw materials of these two kinds of pu-erh tea were large leaves of *Camellia sinensis* var. *assamica* in Yunnan province China, but the manufacture processes of two pu-erh teas were significant difference. After post-fermentation, the content of caffeine in ripened pu-erh tea didn't show significant changes. This process mainly changed the polyphenols, especially the (-)-epigallocatechin gallate, which was mainly degraded during post-fermentation.

Duging the post-feremention, the theabrownin was formed during post-feremention of ripened pu-erh tea. The high molecular weight theabrownin mainly contains alkaloids (caffeine) and tea pigments by the analysis using Curie-point pyrolysis-gas chromatography–mass spectroscopy (18). Usually, it was suggested that theabrowin could be bound with caffeine by hydrogen bonds. There were also many reports about the "cream down" , which was composed of oxidized tea polyphenols bonded with caffeine by hydrogen bonds (19). The *in vitro* bonding reaction promoted the precipitate of caffeine, whereas *in vivo* bonding of theabrowin and caffeine controlled the realeasing of caffeine in gastro-intestinal tract.

The stimulation effects of caffeine restrict the consumption of tea for some consumers who are sensitive to caffeine. The caffeinated drinks have been linked with palpitations, tachycardia and dysrhythmia, which may harm the health of some patients with cardiovascular diseases. In the present study, it was suggested that the interaction of theabrowin with caffeine may lead to a control-release effect on the caffeine, so that decreased the maximum plasma concentration (C_{max}) of caffeine. Furthemore, it was reported that ripened pu-erh tea could promote the fecal excretion of lipids by increasing the small intestinal peristalsis (20,21). The sustained low extent absorption of cafeine contributed to decreasing the side effects of caffeine *in vivo*. These results may provide a proposed mechanism for the low stimulative effects of ripened pu-erh tea during consumption.

Conclusion

In the present study, ripened pu-erh tea decreased the absorption and increased the excretion of caffeine by pharmackointeics study in *vivo* and Caco-2 cells in *vitro*. It was suggested that the unique compounds of ripened pu-erh tea (eg. theabrowin) controlled the release and abosrption of caffeine in ripened pu-erh tea by forming the theabrownin-caffeine binding complex. These results suggest that ripened pu-erh tea may be tolerable for people who are sensitive to caffeine. Our findings provide a theoretical basis for the phenomenon of the mitigation insomnia effects of ripened pu-erh tea in comparison with green pu-erh tea.

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