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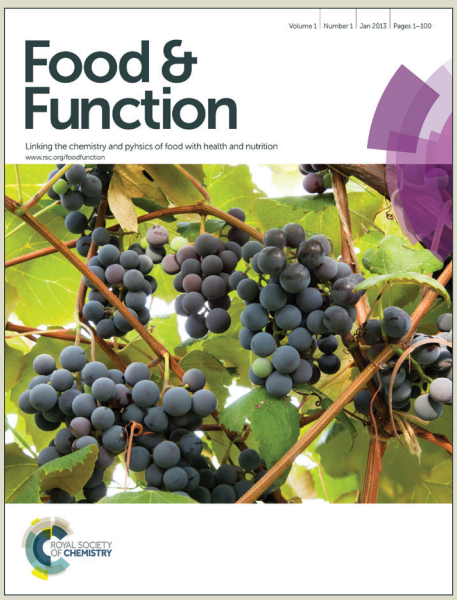

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Absorption of Caffeine in Fermented Pu-er tea is Inhibited in Mice

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Abstract

Caffeine present in number of dietary sources consumed worldwide. Although its pharmacokinetics has been intensively explored, little is known about complexed caffeine (C-CAF) in aqueous extraction of fermented Pu-er tea. The major components of C-CAF are oxidative tea polyphenols (OTP) and caffeine. Furthermore, the C-CAF can be precipitated in low pH solution. After administrating the same amount of total caffeine and comparing the peak level of plasma caffeine with the coffee (contains 0.11 ± 0.01 % C-CAF) group, the results showed that the caffeine/OTP (contains 66.67 ± 0.02 % C-CAF) group and the instant Pu-er tea (contains 23.18 ± 0.02 % C-CAF) group were 33.39 % and 25.86 % lower, respectively. The concentration of the metabolites of caffeine supports the idea that the absorption of the C-CAF was inhibited in mice. Congruent with this result, the amount of caffeine detected in mice excrement showed that more caffeine was eliminated in the caffeine/OTP group and the Pu-er tea group. The locomotor activity tests of mice demonstrated that the caffeine/OTP group and Pu-er tea group were more peaceful than coffee group. Our findings demonstrated that caffeine can be combined with OTP and the absorption of C-CAF is inhibited in mice, thus decreasing the irritation effect of caffeine. This may also be developing as a slow release formulation of caffeine.

Key words: absorption, caffeine, complexed caffeine, fermented Pu-er tea, oxidative tea polyphenols

Introduction

Caffeine (1, 3, 7-trimethylxanthine), a naturally occurring plant xanthine alkaloid[1], was first isolated in the eighteenth century[2]. Caffeine is present in a number of dietary sources consumed worldwide (i.e.,coffee, tea, soft drinks – cola, cocoa beverages, and chocolate bars)[3-5]. About 87 % of the world's population consumes caffeine with an average of 193 mg per day. Among adults 18 years and older, the daily intake ranges between 166 and 336 mg per day[1,6,7]. Caffeine is considered as a stimulant of the central nervous system for its ability to enhance alertness and can inhibit lipid absorption in ovariectomized rats[2,4,8]. The efficacy and side effects of caffeine have been extensively studied[3,6,9-12], caffeine is generally recognized as safe by the Food and Drug Administration. However, excessive caffeine use can result in serious health problems and, in rare cases, death[13,14].

One of the most common side effects of caffeine consumption is sleep loss. Some reported experiments about the negative effects of caffeine on sleep have consistently found that caffeine shortens total sleep time, prolongs sleep latency, and changes the duration of light and deep sleep[15]. Sleep loss is a major reason that people are reluctant to consume caffeinated beverages, namely tea and coffee. Fermented Pu-er tea is a unique fermented tea produced in Yunnan province of China[16], it has been consumed by Chinese people for centuries and has recently become more popular functional beverage in Asian, Europe and America[17,18]. A mass of oxidative tea

polyphenols (OTP) in fermented Pu-er tea are produced during the fermentation process[19,20]. During the stage of fermentation, caffeine content has been found to increase [21]. Contrary to assumption, it is believed that the influnce of fermented Pu-er tea on sleep is significantly less in comparison with other kinds of tea or coffee. Unlike coffee or other kinds of tea, many people can enjoy Pu-er tea before sleep.

The above discrepancy laid the groundwork for us to investigate the effects of caffeine in fermented Pu-er tea. We previously found that caffeine in fermented Pu-er tea can be divided into two kinds as complexed caffeine (C-CAF) and free caffeine (F-CAF), and that the C-CAF content can be evaluated by Low-pH Precipitate Method[22]. In this study, our primary objective was to compare the content of C-CAF in different kinds of tea and coffee samples, and then probe into the material basis of C-CAF formation. On account that mouse stomach acidity has a pH of about 2.0, we postulated that caffeine from Pu-er tea can be precipitated with other components in stomach when administered orally. Resultantly, the absorption of caffeine in Pu-er tea may be unavoidably influenced. To test this hypothesis, our second objective was to investigate the difference between the absorption of caffeine in coffee, fermented Pu-er tea and OTP. Our experiment results demonstrated that: one, fermented Pu-er tea has a larger amount of C-CAF than non-fermented Pu-er tea, green tea, black tea, oolong tea and coffee; two, C-CAF was principally combined with OTP; and three, the absorption of C-CAF in fermented Pu-er

tea was inhibited in mice and the effect of caffeine was influenced significantly. Our findings provide a theoretical basis for the phenomenon of the mitigation insomnia effects of fermented Pu-er tea in comparison with other kinds of tea or coffee.

Materials and methods

Chemicals and reagents. Caffeine was purchased from Aladdin. Theobromine was purchased from Shanghai Yuanye biological technology co., LTD. Paraxanthine, theophylline and sodium dihydrogen phosphate were purchased from Sigma-Aldrich. 8-chlorotheophylline was purchased from Tokyo chemical industry co., LTD. Methanol used in the mobile phases were of HPLC-grade reagent purchased from Tedia Co. Inc.. Deionized water was prepared using the Purifier (FST-UV-20, Shanghai Fushite instrument equipment Co., Ltd. Shanghai, China) and was degassed by ultrasonic cleaning machine (SK-18TC, Shanghai Kudos ultrasonic instrument Co., Ltd. Shanghai, China).

Samples and preparation of HPLC infusion. Fourteen Chinese tea samples of various types, including three fermented Pu-er tea, two Non-fermented Pu-er tea, three black tea, two oolong tea, and four green tea, which were made into aqueous extract powder. Moreover, three coffee samples, one instant essence of Pu-er tea (Deepure, Yunnan tasly deepure biological tea group co., LTD.), one OTP, one TPS, one Pu-er tea theabrownin (PTTB), and one Pu-er tea polysaccharide (PTPS) sample were also made into aqueous

extract power. Supporting information of all samples is listed in **Table 1**. The preparation process of OTP, TPS, PTPS and PTTB was showed in the "Supplementary Experimental Procedures" of Electronic Supplementary Information.

Preparation of HPLC infusion: 800mg of the sample was dissolved in 40 mL ultrapure water and was shocked by Vortex Mixer for 2 min and then was centrifuged at 800 ×g for 10 min. The resulting supernatant was diluted 10-folds and filtered through a 0.45μm membrane and subjected to HPLC to determine the content of caffeine and its metabolites of the sample. In addition, the 20 g/L sample solution would be used for Low-pH Precipitate Method and Chloroform Extraction as described below.

HPLC-VWD analysis. A 50 μL sample was analyzed using an autosampler (G1329B, 1260ALS, Agilent, USA), a ultraviolet detector (G1314F, 1260VWD, Agilent, USA) at wavelength 274 nm, and HPLC pump (G1311B, 1260Quat Pump, Agilent, USA) at 0.6 mL/min flow at 30℃ (G1316A, 1260TCC, Agilent, USA) through a C18 ODS column (ZORBAX SB-C18 4.6mm × 250mm, 5 Micron, Agilent, USA) with a guard column (ZORBAX Eclipse Plus-C18, 4-Pack, Analytical Guard Column, 4.6mm×12.5mm, 5-Micron, Agilent, USA). The mobile phase was 73 % 0.1 mol/L sodium dihydrogen phosphate and 27 % methanol, filtered through a 0.45 micron filter. Concentrations of caffeine and its metabolites were determined in mg/L for all of the samples[3].

Determination of C-CAF content of the samples by Low-pH Precipitate

Method. The C-CAF content of the samples was evaluated by Low-pH Precipitate Method as previously described[23]. In brief, the poly-phenolic hydroxyl contained components were precipitated under acidic conditions (pH ≤ 2) and then dissolved with alkaline solution. Conveniently, if caffeine was combined with the Poly-phenolic hydroxyl contained components, the C-CAF content of the samples can be determined in the precipitate dissolved solution by HPLC.

Sample preparation: Hydrochloric acid was added to 12mL of 20 g/L sample solution for adjusting acidity to pH 1.5 and was shocked by Vortex Mixer for 2min and centrifuged at 1600 × g at 25 ℃ for 30 min. The precipitate and supernatant were separated and the precipitate was weighed by sensitive balance. The precipitate was dissolved NaOH solution (pH = 10). Then, the supernatant and precipitate solution were diluted with deionized water to 13mL. The caffeine content of the supernatant and precipitate was determined by HPLC.

Checkout of C-CAF content of the samples through chloroform extraction. The linear regression equation of the balance line of caffeine distribution between water and chloroform was $y = 15.02x$ at 25 ℃. X and y mean that caffeine concentration (g/L) of aqueous phase and chloroform phase respectively when the distribution achieves equilibrium. The correlation coefficient R = 0.9942. It has no significant effect on the caffeine extraction between pH = 2.28 ~ 11.37 when $x < 0.6$ g/L[24]. To checkout content of C-CAF

of the samples, we determined caffeine distribution ratio of the samples between chloroform phase and aqueous phase.

Sample preparation: 5mL chloroform was added to 5 mL of 20 g/L sample solution and was shocked by Vortex Mixer for 2 min and centrifuged at 800 ×g at 25 ℃ for 15 min. Then, the aqueous phase solution was diluted 10 folds with deionized water. The caffeine content was determined by HPLC.

Mice. Healthy virgin inbred BALB/c mice (7–8 weeks old) composed of male and female in equal numbers from Nanjing Peng-sheng biotechnology Co., Ltd, China, were used in the animal experiments. Mice were maintained in a controlled environment (12 h light/12 h dark cycle; humidity 50–60 %; ambient temperature 24°C ± 1°C) and were administered standard laboratory food and water *ad libitum.* All mice experiments were performed in the animal facility according to institutional guidelines and were approved by the Institutional Animal Care and Use Committee of Yunnan Agricultural University. Adverse events were not observed.

Group designations and caffeine administration. The BALB/c mice composed of male and female in equal numbers were divided at random into coffee group, fermented Pu-er tea group, and caffeine added OTP group. Coffee (85.91 g/L), fermented Pu-er tea (31.77 g/L) and OTP (31.77 g/L) with additional caffeine were prepared in deionized water (the caffeine content of the three samples are equivalent to 2.5 g/L) and administered via intragastric administration (0.2 mL/10g). The caffeine dose (50 mg/kg) was selected based

155 on its pharmacokinetic profile in mice compared with humans, which is well
156 below the lethal dose of approximately 10 g in humans [11]. Caffeine doses were
157 also based on the finding that 50 mg/kg of caffeine in rodents corresponds to
158 approximately 10 cups of coffee for humans, but caffeine is metabolized up to
159 6 times faster in mice than in humans[5,25]. The mice were feed-deprived for 12
160 hours before the intragastric administration.

161 ***Levels of caffeine and its metabolites in plasma.*** Levels of caffeine and its
162 metabolites in plasma were determined by HPLC as described by Youngberg[3]
163 et al. with modifications. In brief, the blood samples were taken at various
164 intervals (5, 10, 20, 40, 60, 120, 240, 360, 480 min) after the caffeine
165 administration. As the internal standard, 3.33 mg/L of 8-chlorotheophylline was
166 added to each sample. 150 µl samples of subject plasma were extracted in 1
167 mol/L HCl and 3 mL methylene chloride. The organic layer was evaporated to
168 dryness and reconstituted in 0.1 mol/L HCl solution. Plasma levels of caffeine
169 and its metabolites were determined by HPLC with the same chromatographic
170 condition as "*HPLC-VWD analysis*" part. For caffeine, paraxanthine,
171 theophylline and theobromine in plasma: the limit of quantitation (mg/L) was
172 0.05, 0.10, 0.105 and 0.035; the limit of detection (mg/L) was 0.025, 0.045,
173 0.060, 0.015; the response factor was 1.005, 0.456, 0.514 and 0.906; and the
174 overall extraction recovery from human plasma was 85.28 ± 2.31 %, 57.13 ±
175 0.87 %, 60.10 ± 0.39 % and 72.84 ± 0.87 %, respectively. The recovery for the
176 internal standard (8-chlorotheophylline) was 90.89 ± 1.07 %. The precision and

accuracy for the analytes were within the acceptable range (< 7 %). The assay validation was showed in the “supplemental data” of Electronic Supplementary Information.

Excrement sample extraction. The excrement samples were taken 8 hours after the caffeine administration, and then dried and ground to powder. 5 mL methanol (70 %) was added to a 200 mg powder sample, shocked by Vortex Mixer for 2min, and then extracted by ultrasound-assisted for 30 min at room temperature. Finally, the caffeine concentration was determined by HPLC.

Evaluation of locomotor activity. Locomotor activity was monitored with a mouse activity monitor system (ZZ-6, Taimeng, Chengdu Technology & Market Co. Ltd., Chengdu, China), which monitored the horizontal (locomotion) and vertical (rearing) movements of the mice. The individual compartments (L=15; W=12; H=10 cm) were put in a dimly lit and quiet room. The mice were feed-deprived for 12 hours before the experiment. Groups of female BALB/c mice were first thoroughly habituated to the test environment over a 30 min period. Then, they were removed from the open field, administrated with coffee, fermented Pu-er tea, and caffeine added OTP aqueous solution (which were equivalent to caffeine at 5 mg/kg) or water respectively via intragastric administration (0.2 mL/10g), and replaced in the compartments for an additional 90 min. The 5 mg/kg test dose of caffeine was chosen on the basis of previous dose-effect studies performed in our laboratory. This dose is a little higher than the threshold of stimulant effect of caffeine for the female mice.

Locomotor activity was recorded during the 90 min following the caffeine administration. The floor and walls of the chamber were cleaned with ethanol (70%) and dried with paper towels between each mouse exposure[26-28].

Statistical analyses. All values were presented as mean ± the standard error of the mean (SEM). Differences within groups were analyzed with repeated measures one-way ANOVA, Two-tailed $p < 0.05$ was considered to be statistically significant. All analyses were performed using SPSS 17.0 (Chicago, IL, USA) and GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

C-CAF content of the samples. Previous research has shown that tea contains theobromine and theophylline in addition to caffeine[29]. Therefore, in the current study, we set out to observe plasma levels of caffeine and its metabolites, by initially evaluating the content of caffeine, theophylline, theobromine and paraxanthine of the samples (**Supplemental Table 1**). There was a large amount of caffeine in the tea samples, whereas, there was slight amount of caffeine in the OTP. In addition, the tea and coffee samples contain small amounts of theophylline and theobromine. There was a relatively high level of theobromine in the fermented Pu-er tea samples, but only trace amounts of paraxanthine in the tea and coffee samples.

To evaluate and compare the C-CAF content of all samples, we next determined the C-CAF content by Low-pH Precipitate Method (**Table. 1**). Nearly a quarter of the caffeine in fermented Pu-er tea was C-CAF. What's

more, about three-quarters of the caffeine in the caffeine added OTP sample was C-CAF. Only about one-tenth of caffeine in the black tea samples, however, was C-CAF. In the non-fermented Pu-er tea, oolong tea, green tea and caffeine added TPS there was just a moderate level of C-CAF. Curiously, the C-CAF levels of PTPS and caffeine added PTTB were also high. In addition to fermented Pu-er tea, the C-CAF percent of the caffeine added OTP, PTPS and caffeine added PTTB samples were also obviously greater than the percent of precipitate volume (**Table. 1 and Supplemental Table 2**). These results indicate that, compared to the other samples, fermented Pu-er tea, caffeine added OTP, PTPS, and caffeine added PTTB samples contained more C-CAF, and caffeine in these samples abounded in the precipitate components; while there was hardly any C-CAF in the coffee samples.

To further characterize the combination of caffeine with other components of the samples, the fermented Pu-er tea, OTP and coffee samples were examined through Chloroform Extraction (**Fig. 1**). The OTP were mainly distributed in the aqueous phase. The caffeine concentration ratio of the Chloroform phase to aqueous phase was determined with significant differences ($P < 0.001$) among the coffee, fermented Pu-er tea and OTP samples. For the fermented Pu-er tea and OTP samples, the balance of caffeine distribution between water and chloroform was shifted severely. The caffeine concentration ratio of the Chloroform phase to aqueous phase in descending order was as follows: coffee samples, fermented Pu-er tea

243 samples and OTP samples. These results indicate that caffeine in the OTP and
244 fermented Pu-er tea samples was combined with OTP which belonged to the
245 aqueous phase.

246 ***Effect of pH on the formation of caffeine contained precipitate.*** To further
247 probe into whether or not fermented Pu-er tea aqueous extract or OTP can be
248 precipitated in the condition of mouse stomach, we examined the association
249 between the formation of precipitate and solution acidity (**Fig. 2 A, B**). Plenty of
250 precipitate was formed at pH = 1.5 in the OTP and fermented Pu-er tea
251 solution (**Fig. 2 A**) and the C-CAF percent of the OTP and fermented Pu-er tea
252 samples was high (**Fig. 2 B**). Compared to the pH of 1.5, in spite of the
253 precipitate quantity, the percentage of C-CAF was less at pH 3.5, but obvious
254 precipitate was still formed. Consequently, the fermented Pu-er tea and OTP
255 might be precipitated by internal conditions of the mouse stomach.

256 ***Plasma levels of caffeine and its metabolites.*** The results above suggest
257 that the caffeine in fermented Pu-er tea was combined with OTP and the
258 C-CAF may be precipitated in mouse gastric juice. Nearly the same as pure
259 caffeine, there is hardly any C-CAF in coffee. Therefore, coffee was selected
260 as a control group to investigate whether the absorption of caffeine was
261 influenced by combining with OTP. Following the coffee, fermented Pu-er tea
262 and caffeine added OTP administration, plasma levels of caffeine, and its
263 metabolites were determined at different time points (**Fig. 3 A, B, C, D**).

264 Following the sample solutions administration, plasma levels were not

significantly different 5 or 10 min later. However, compared to the coffee group, plasma caffeine levels of the fermented Pu-er tea group and the caffeine added OTP group were significantly reduced at 20 min and 40 min after ingestion ($P<0.05$). Moreover, there were significant differences of plasma caffeine levels between the coffee group and the caffeine added OTP group at 60 min and 120 min after ingestion ($P<0.01$). There were not significant differences between the coffee group and the fermented Pu-er tea group 40 min after ingestion (**Fig. 3 A**).

For paraxanthine and theophylline, the plasma levels of the fermented Pu-er tea group and the caffeine added OTP group were higher than the coffee group in the early stage (5 min to 60 min). In contrast, the plasma levels of the two groups above were lower than the coffee group after 120 min (**Fig. 3 B, C**). Noteworthy, the plasma theobromine levels of the fermented Pu-er tea group were far higher than the other two groups before 360 min ($P < 0.05$) (**Fig. 3 D**). Compared to the coffee group, the plasma theobromine levels of the caffeine added OTP group were higher in the early stage (5 min to 60 min), with the most significant difference occurring 10 min after ingestion ($P<0.01$). Interestingly, the plasma levels of theobromine of the caffeine added OTP group were also lower than the coffee group after 120 min.

Excrement levels of caffeine. Given that the absorption of C-CAF in fermented Pu-er tea and OTP was decreased within a prescribed time limit, we speculated that the absorption of C-CAF must be either delayed or inhibited in

mice. To ascertain the cause of this, the excrement levels of caffeine were examined (**Fig. 4**).

Excrement levels of caffeine from the fermented Pu-er tea group and caffeine added OTP group were significantly higher than that of the coffee group ($P < 0.0001$). The result indicates that more caffeine was excreted in the fermented Pu-er tea group and the caffeine added OTP group compared to the coffee group. In all probability, the absorption of C-CAF was inhibited.

Locomotor activity levels. The result above suggested that caffeine can be combined with OTP, and the absorption of C-CAF can be inhibited in mice. To better understand the biological significance of that, we then performed another experiment for monitoring the locomotor activity of mice to elucidate the effects of C-CAF (**Fig. 5**).

The locomotor activity levels of the water and caffeine added OTP groups were lower than the coffee group, significantly ($P < 0.05$). The fermented Pu-er tea group was also lower than the coffee group ($P = 0.14$). These results indicate that the effect of caffeine in vivo was inhibited in the fermented Pu-er tea group and the caffeine added OTP group.

Discussion

To clarify the absorption of C-CAF in mice, it is necessary to discuss the total caffeine, C-CAF and caffeine metabolites content of the samples, and probe into which components can combine with caffeine. Previous studies[34-36] have reported that total sugar (14.47-27.62 %), caffeine (2.35-10.43 %) and

309 catechins (29.86-78.66 %) were found to be the main chemical components of
310 tea cream in the green tea concentrate. Furthermore, the effect of caffeine on
311 tea cream and haze of tea infusions is dependent on the presence of sufficient
312 substances within pyrogallol groups[37]. Our previous study[22] also showed that
313 the caffeine in fermented Pu-er tea can be categorized into F-CAF and C-CAF.
314 However, fermented Pu-er tea contained trace free tea catechins. The major
315 catechins were transformed to OTP due to the specific feature of the catechin
316 – a strong anti-oxidant and easily oxidated in the fermentation process.
317 Theaflavins, thearubigins and theabrownins are the main complex phenolic
318 compounds deriving from the oxidation of catechins and their gallates during
319 the fermentation process. Theaflavins undergo further oxidation during
320 fermentation to form more polymerized thearubigins, and then condensed
321 theabrownins[18,38]. The OTP still contained a large amount of benzene groups
322 even though their chemical structure is not cleared, because the original
323 catechins retain at least one benzene ring and the absorption curve of the OTP
324 has characteristic peaks of benzene. The OTP was charactered as polymeric
325 organic acid, which can be precipitated by adjusting the pH value to 2.
326 Coincidentally, an increased level of C-CAF in tea is associated with the
327 increased degree of oxidization or fermentation during the processing of tea.
328 There is abundant OTP in fermented Pu-er tea, which is closely associated
329 with a high level of C-CAF[39]. Accordant with previous studies[22], the C-CAF
330 content of fermented Pu-er tea is far higher than black tea, green tea, and

instant coffee.

In order to investigate the material basis of the C-CAF we attempted to separate PTPS and PTTB – two main components in Pu-er tea[33]. In doing so, we found that PTTB can be precipitated with PTPS during the alcohol sedimentation process, and PTPS and PTTB are difficult to separate. In essence, OTP is the principal component of the PTPS and PTTB samples. The significantly lower C-CAF content of PTTB to that of OTP may result from the fact that PTTB was mixed with other components, TPS for example. As another major component in the extraction of tea, TPS is a kind of poly-sacchride that may be, during the fermentation process, hydrolyzed to oligosaccharides and monosaccharides. In keeping with this interpretation, results of Chloroform Extraction for caffeine are consistent with the results of the Low-pH Precipitate Method. These results highlight the importance of OTP in the formation of C-CAF and demonstrated that OTP is the main material basis for the formation of C-CAF in fermented Pu-er tea.

As the major stimulant compound to human central nervous system[30], caffeine is known to form complexes by self-association and with tea catechins. The Crystalline structure of the complexes of (-)-catechin (CA) and (-)-catechin-3-*O*-gallate (Cg) with caffeine have shown that CA formed a 1 : 1 complex with caffeine by intermolecular hydrogen bonds, whereas Cg formed a 2 : 4 complex with caffeine by face-to-face and offset π-π interactions and intermolecular hydrogen bonds[31,32]. The ^{1}H-NMR spectra data indicated that

353 the chemical shift for caffeine between reference sample (free caffeine) and
354 the complex of OTP & caffeine sample changed little (Supplemental Figure 2,
355 Supplemental Table 3 and Supplemental Figure 3). These results suggest that
356 OTP and caffeine formed a complex by intermolecular forces but without
357 covalent bonds combination.

358 Moreover, we should note that the effect of pH on tea solids extraction
359 yield was significant. Total solids extracted between pH levels 2 to 11 varied
360 from 270 to 290 g/kg tea, but figures were doubled at pH 1.1[40]. One can
361 speculate that pH is also a key factor to the precipitate quantity of the
362 fermented Pu-er tea and OTP aqueous solution. Considering that caffeine can
363 be combined with OTP and can form a precipitate with OTP in a Low-pH
364 solution, we sought to determine if the C-CAF can be precipitated in the
365 condition of mouse stomach. For this purpose, we examined the precipitate
366 quantity and percent of C-CAF of the samples at the pH similar to the stomach.
367 The results showed that the caffeine in fermented Pu-er tea and OTP samples
368 might be precipitated in the condition of mouse stomach. In addition, it's worth
369 noting that the content of C-CAF in the coffee samples was very low.

370 After ascertaining the above results, we examined whether the absorption
371 of the C-CAF was influenced in mice. To address this issue, we investigated
372 the plasma levels of caffeine and its metabolites after the mice were
373 administrated caffeine contained samples. In agreement with previous reports[4],
374 our study showed that the mice plasma caffeine concentration of the coffee

group peaked 20min after the ingestion of caffeine (50 mg/kg). In marked contrast, the plasma levels of caffeine of the fermented Pu-er tea and caffeine added OTP group varied marginally between 10 and 40 min, and were significantly lower than the coffee group. The simplest explanation for this observation is that the absorption of caffeine in fermented Pu-er tea and OTP was reduced, significantly.

Caffeine from coffee or other beverages is absorbed by the small intestine within 45 min of ingestion and moves through cellular membranes with the same efficiency that it is absorbed and circulated to tissues of the body[41]. It is eliminated by first-order kinetics[42]. Caffeine is metabolised by CYP enzymes (de-methylation), xanthine oxidase (formation of uric acid metabolites) and/or N-acetyltransferase (acetylation) in the liver, and through enzymatic action results in three metabolites: paraxanthine, theophylline, and theobromine[43]. In contrast to caffeine, the plasma levels of paraxanthine and theophylline in the fermented Pu-er tea group and caffeine added OTP group were higher than the coffee group in the early stage (5min to 60min), but were lower than the coffee group after 120 min. Nevertheless, the paraxanthine and theophylline intake from the fermented Pu-er tea group and caffeine added OTP group is not more than the coffee group (**Table 1**). One postulate for the above results is that some components (probably OTP) influenced the distribution or metabolism of caffeine in the mice. However, owing to more caffeine was absorbed and metabolized by the mice in the coffee group, the plasma levels

of paraxanthine and theophylline in the fermented Pu-er tea group and caffeine added OTP group were lower than the coffee group after 120 min. As a result of the dramatically higher theobromine content of fermented Pu-er tea in comparison to the coffee and OTP groups (**Table 1**), the fermented Pu-er tea group plasma levels of theobromine were far higher than the other two groups before 360min.

To assist in determining if the significantly lower plasma levels of caffeine found in the fermented Pu-er tea and caffeine added OTP group were the result of caffeine metabolism, we tested the mice's excrement levels of caffeine. Our findings confirmed that the absorption of C-CAF was inhibited in mice and was excreted, in part. The popularity of investigating caffeine has generated more interest from scientists of late due to the potentially harmful effects it has on our health[2]. Caffeine is a non-selective antagonist for adenosine receptors[27]. Several studies confirm that the activation of adenosine receptors $A_1$and A_{2A}, as well as the regulation of adenosine production and degradation, is essential for sleep induction and proper control of the sleep-wakefulness cycle[44]. The results of locomotor activity of mice indicate that the irritation effect of caffeine was inhibited when complexed with OTP.

To our knowledge, this is the first report that investigated the absorption of C-CAF in mice. Several main findings were obtained that might advance the theoretical framework by potentially illustrating why different sources of caffeine have different effects on humans. If the total caffeine intake is

consistent, the high level of C-CAF may be beneficial for people with sensitivity to caffeine to reduce the side effects of caffeine, such as insomnia. Moreover, this may be meaningful for developing some low F-CAF level products.

Conclusions

In conclusion, our results showed that OTP formed with caffeine to become the C-CAF contained in fermented Pu-er tea. The absorption of this C-CAF is inhibited in mice and may be the same in humans. What's more, the effect of caffeine was influenced significantly when it was combined with OTP. Taken together, this offers insight into the influences of fermented Pu-er tea on sleep loss are diminished in comparison with other forms of caffeine and/or caffeine containing products.

Associated content

Abbreviations Used

C-CAF, complexed caffeine; F-CAF, free caffeine; OTP, oxidative tea polyphenols; PTPS, Pu-er tea polysaccharide; PTTB, Pu-er tea theabrownin; TPS, tea polysaccharides.

Acknowledgments

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443 2009AB021).

444 **Supplementary Material**

445 Refer to Web version on PubMed Central for the preparation process of OTP,
446 TPS, PTPS and PTTB; the content of caffeine, theophylline, theobromine and
447 paraxanthine of the samples (Supplemental Table 1); the percent of precipitate
448 volume of the samples (Supplemental Table 2); “Assay Validation” for Levels of
449 caffeine and its metabolites in plasma (Supplemental Figure 1); and ^{1}H NMR
450 spectra data for caffeine (Supplemental Figure 2, Supplemental Table 3 and
451 Supplemental Figure 3).

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457 **Notes**

458 The authors declare no competing financial interest.

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TABLE 1. The content and percent of C-CAF of the samples[1]

sample type	sample name	F-CAF, *mg/L*	C-CAF, *mg/L*	C-CAF, *%*
fermented pu-erh tea	Gold bud tribute pu-erh tea	1716.13±0.93	486.54±3.81	22.09±0.14
	TAETEA pu-erh tea (7592)	1318.77±0.48	378.47±2.68	22.30±0.13
	Brick pu-erh tea	1370.85±0.22	267.63±1.13	16.33±0.06
non-fermented pu-erh tea	Moonlight White pu-erh tea	2530.20±0.50	60.23±0.47	2.32±0.02
	Bangwei pu-erh tea	1179.72±1.25	11.27±0.20	0.94±0.02
black tea	Changning black tea(first grade)	1100.19±1.41	115.78±0.64	9.52±0.04
	MAKEMY black tea	1830.36±1.22	261.60±0.52	12.50±0.02
	Dianhong black tea	1443.38±0.11	118.37±0.49	7.58±0.03
oolong tea	Tieguanyin oolong tea	1033.37±7.51	6.29±0.47	0.60±0.04
	Da Hung Pao oolong tea	1460.97±4.83	69.08±0.51	4.52±0.05
green tea	Longjing green tea	1474.15±0.46	13.63±0.24	0.92±0.02
	Biluochun green tea	1399.68±2.39	20.78±0.84	1.46±0.06
	Huilong green tea	1387.57±4.29	8.80±0.06	0.63±0.00
	Yunnan green tea	1411.13±1.57	10.91±0.27	0.77±0.02
coffee	Nescafe coffee	550.58±0.40	0.63±0.06	0.11±0.01
	Maxwell House coffee	664.36±0.19	0.98±0.02	0.15±0.00
	Yunnan Pasteral coffee	702.01±0.45	0.76±0.03	0.11±0.00
instant fermented pu-erh tea	Deepure instant essence of pu-erh tea	1162.04±0.65	350.63±0.62	23.18±0.02
OTP	OTP	45.92±0.44	146.95±0.61	76.19±0.16
PTPS	PTPS	262.13±0.73	165.80±2.06	38.74±0.34
mixture of TPS and caffeine	caffeine added TPS	1085.51±0.99	34.92±0.07	3.12±0.01
mixture of OTP and caffeine	caffeine added OTP	415.83±0.58	831.85±0.42	66.67±0.02
mixture of PTTB and caffeine	caffeine added PTTB	825.93±0.23	317.33±0.24	27.76±0.02
caffeine	caffeine	1234.68±0.63	0.70±0.05	0.06±0.00

[1]Data were presented as mean ± SEM of three independent experiments. C-CAF, complexed caffeine; F-CAF, free caffeine; OTP, oxidative tea polyphenols; PTPS, Pu-er tea polysaccharide; PTTB, Pu-er tea theabrownin; TPS, tea polysaccharides.

Figures

FIGURE 1

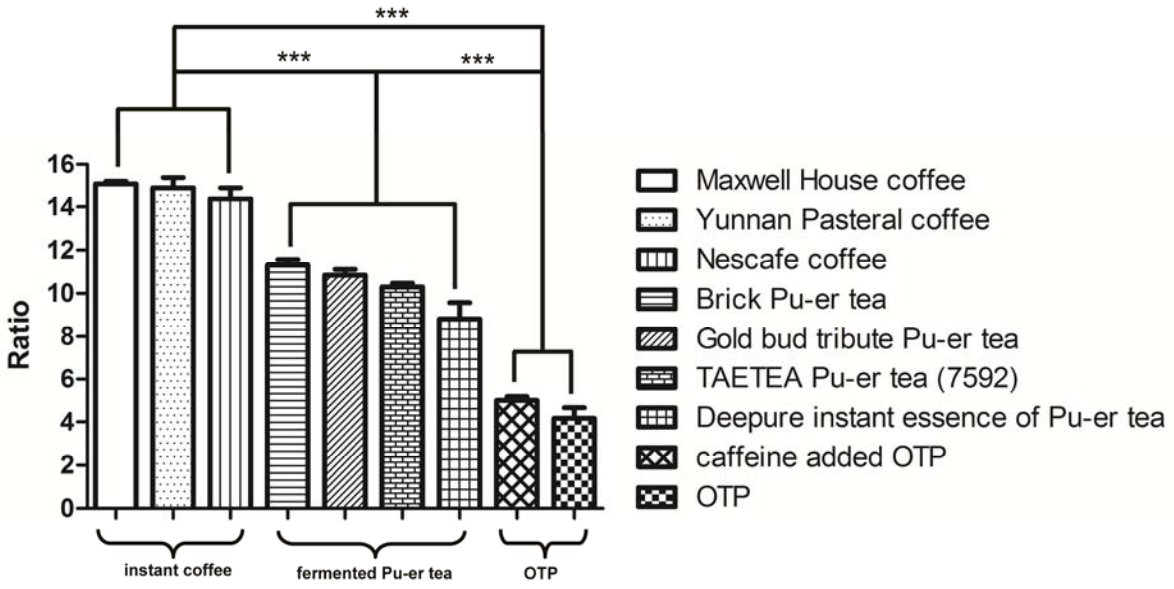
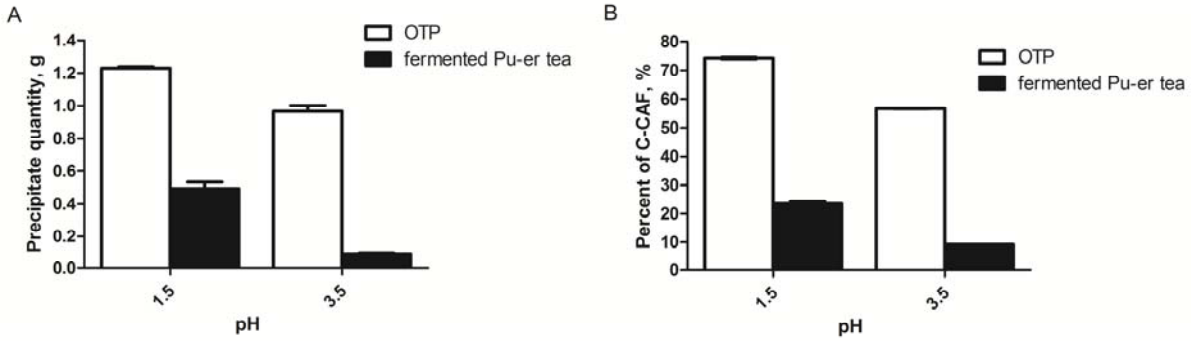


FIGURE 2



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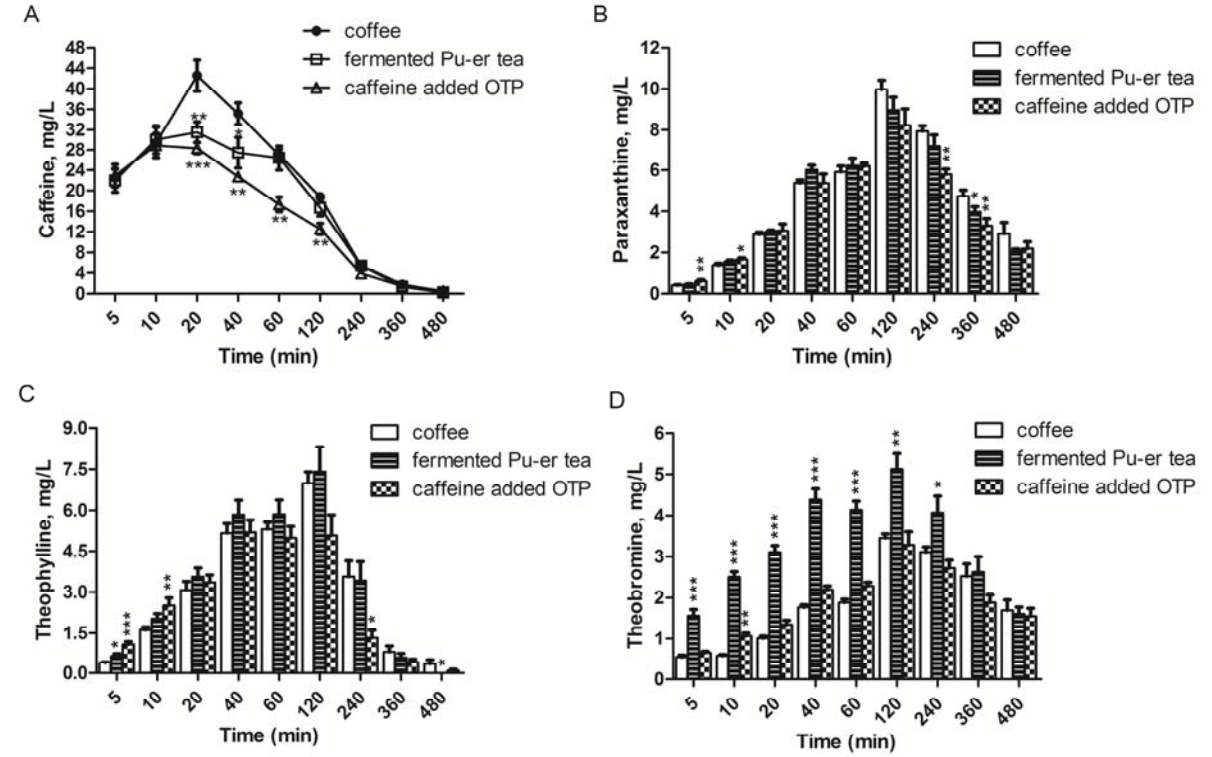

FIGURE 4

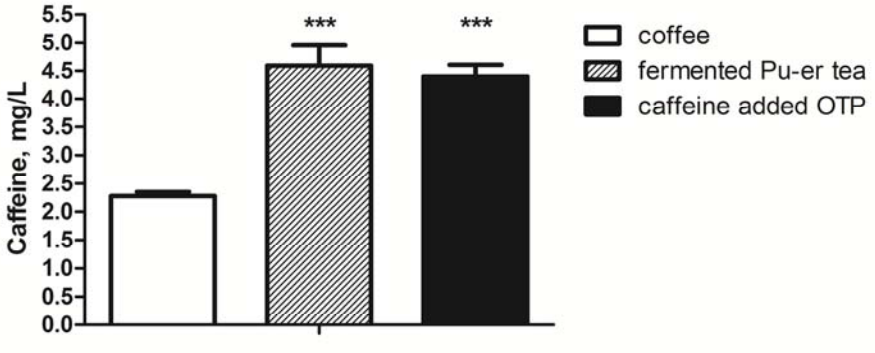

FIGURE 5

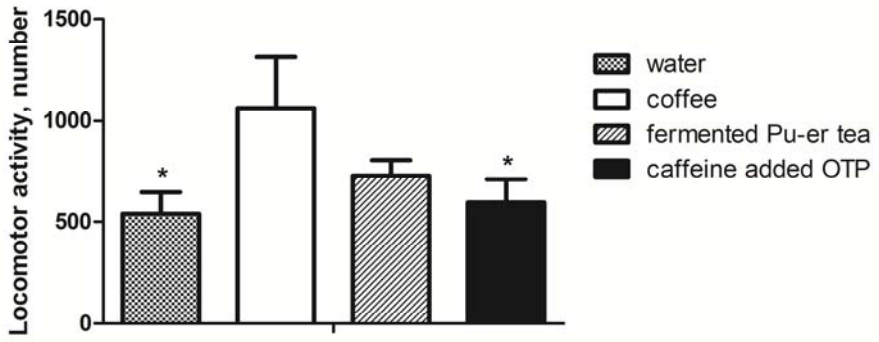

Figure captions

FIGURE 1. Caffeine concentration ratio of Chloroform phase to aqueous phase. Data were presented as mean ± SEM and were analyzed with repeated measures (n=4) one-way ANOVA, Two-tailed $p<0.05$ was considered to be statistically significant (***P<0.001 vs. coffee samples). OTP, oxidative tea polyphenols.

FIGURE 2. Effect of pH on the formation of precipitate. Data were presented as mean ± SEM and were analyzed with repeated measures (n=3) one-way ANOVA. OTP, oxidative tea polyphenols.

FIGURE 3. Plasma levels of caffeine (A), paraxanthine (B), theophylline (C) and theobromine (D). Data were presented as mean ± SEM. Differences within groups at different time points were analyzed with repeated measures (n=6) one-way ANOVA, Two-tailed $p<0.05$ was considered to be statistically significant. (*P<0.05, **P<0.01 and ***P<0.001 vs. coffee group). OTP, oxidative tea polyphenols.

FIGURE 4. Excrement levels of caffeine. Data were presented as mean ± SEM. Differences within groups were analyzed with repeated measures (n=9) one-way ANOVA, Two-tailed $p<0.05$ was considered to be statistically significant. (***$P<0.001$ vs. coffee samples). OTP, oxidative tea polyphenols.

FIGURE 5. Locomotor activity levels after caffeine administration. Data were presented as mean ± SEM. Differences within groups were analyzed with repeated measures (n=6) one-way ANOVA, Two-tailed $p<0.05$ was considered to be statistically significant. (*$P<0.05$, vs. coffee group). OTP, oxidative tea polyphenols.