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RESEARCH ARTICLE

Puerarin exhibits greater distribution and longer retention time in neurons than astrocytes in a co-cultured system

Shu-Yong Wei[1, 2], Jie Tong[1], Qiang Xue[1], Fang-hong Shang[1], Yan-jun Li[1], Yang Liu[1], Bin-bin Feng[3], Xiao-yu Xu[1, *]

1 Pharmaceutical Sciences College & Chinese Medicine College of Southwest University; Chongqing Engineering Research Center for Pharmacodynamics Evaluation, Chongqing, China
2 Rongchang Campus of Southwest University, Chongqing, China
3 Chongqing Three Gorges Medical College, Chongqing, China

Correspondence to:
Xiao-yu Xu, xuxiaoyu@swu.edu.cn.

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Abstract

The phytoestrogen puerarin has been shown to protect neurons and astrocytes in the brain, and is therefore an attractive drug in the treatment of Alzheimer's disease, Parkinson's disease and cerebral ischemia. Whether puerarin exhibits the same biological processes in neurons and astrocytes *in vitro* has rarely been reported. In this study, cortical neurons and astrocytes of newborn Sprague-Dawley rats were separated, identified and co-cultured in a system based on Transwell membranes. The retention time and distribution of puerarin in each cell type was detected by fluorescence spectrophotometry and fluorescence microscope. The concentration of puerarin in both co-cultured and separately cultured neurons was greater than that of astrocytes. Puerarin concentration reached a maximum 20 minutes after it was added. At 60 minutes after its addition, a scant amount of drug was detected in astrocytes; however in both separately cultured and co-cultured neurons, the concentration of puerarin achieved a stable level of about 12.8 ng/mL. The results indicate that puerarin had a higher concentration and longer retention time in neurons than that observed in astrocytes.

Key Words: *nerve regeneration; puerarin; in vitro experiments; co-culture; neurons; astrocytes; Transwell; neonatal rats; neural regeneration*

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Introduction

As the main active constituent of the plant *Pueraria lobata* (Willd.) Ohwi (Fabaceae), puerarin (Pur) is considered to be one of the most important phytoestrogens and has been widely used in China in clinical treatment (Wei et al., 2014). The neuroprotective effects of Pur have been established *in vivo* in Alzheimer's disease (Xu and Zhao, 2002; Xu et al., 2004), Parkinson's disease (Li et al., 2003; Zhu et al., 2010), and cerebral ischemia (Xu et al., 2007; Chang et al., 2009; Wu et al., 2009), and *in vitro* on primary cultured neurons (Li et al., 2003, 2010; Zou et al., 2013), gliocytes (Zheng et al., 2012), and other cell lines (Lin et al., 2010; Xing et al., 2011; Zhang et al., 2012; Zhu et al., 2012). The potential mechanisms of these neuroprotective effects include alleviating mitochondrial oxidative stress (Xu and Zhao, 2002; Li et al., 2010; Zou et al., 2013), anti-apoptosis (Li et al., 2003; Chang et al., 2009; Wu et al., 2009; Lin et al., 2010; Zhu et al., 2010), inhibiting excitatory toxicity (Xu et al., 2004, 2007), and promoting cell proliferation (Xing et al., 2011; Zhang et al., 2012; Zhu et al., 2012).

Although various studies have indicated neuroprotective effects of Pur, the difficulty in completely separating each kind of neurocyte and subsequently detecting individual Pur concentration *in vivo* means that the target of Pur in the brain is still unclear. Our team has reported Pur concentration in the cerebrospinal fluid of Sprague-Dawley (SD) rats (Wang et al., 2012), but until now, there has been no report on the different distributions and retention time lengths of this drug in cortical neurons and astrocytes. In the present study, we established a system to co-culture cortical neurons and astrocytes. The different distributions and retention time lengths of Pur in these two kinds of neurocytes were analyzed with the purpose of providing further insight into the mechanism underlying the neuroprotection of Pur.

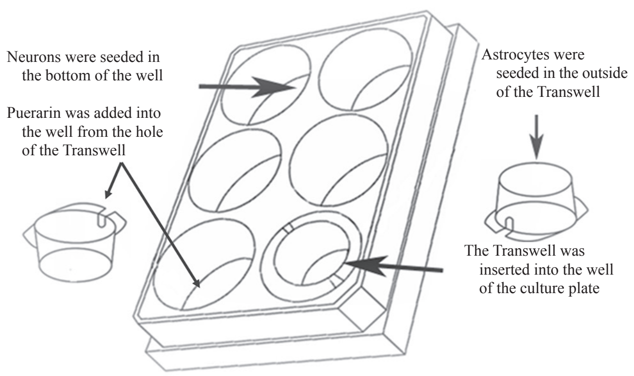

Figure 1 The process of co-culturing neurons and astrocytes.

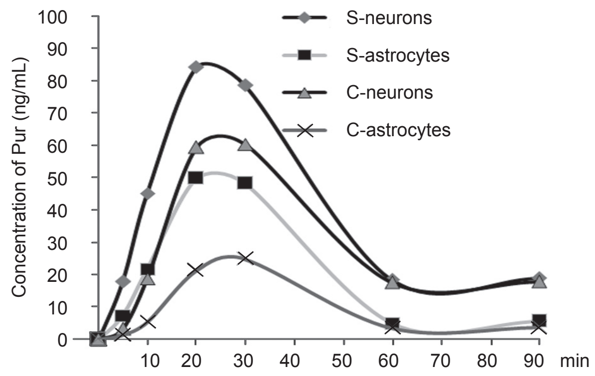

Figure 3 Concentration of puerarin (Pur) in tested cells after co-cultured with Pur for 0–90 minutes.
S-neurons and S-astrocytes: Separately cultured neurons and astrocytes; C-neurons and C-astrocytes: co-cultured neurons and astrocytes.

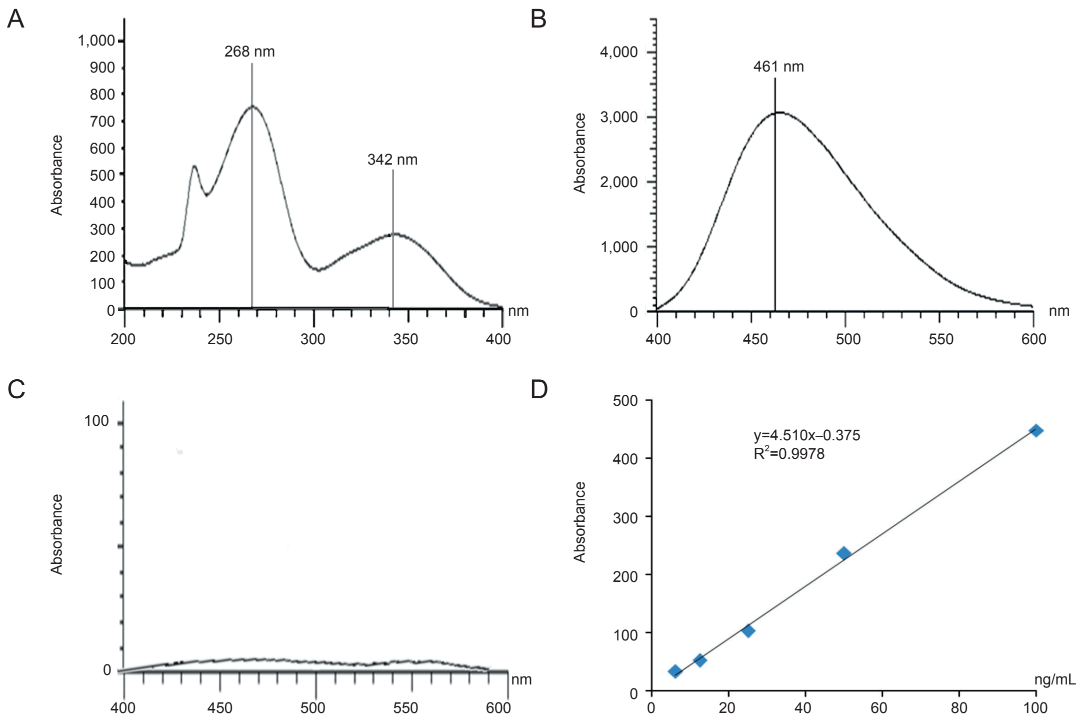

Figure 2 Absorption spectrum and standard curves of puerarin (Pur).
(A) The excitation spectrum of Pur; (B) the emission spectrum excited at 342 nm; (C) the absorption of cell extraction in detected condition; (D) the standard curve of Pur (R^2 = 0.9978).

Materials and Methods

Animals

SD rats aged 24–72 hours were used to culture neurons and astrocytes. Rats were obtained from the Experimental Animal Center, Chongqing Research Institute of Traditional Chinese Medicine (China; production license No. SCXK (Yu) 02007-0006). Temporary rearing was performed at the Experimental Animal Center, College of Pharmaceutical Sciences & College of Chinese Medicine, Southwest University (Chongqing, China; experimental animal use license No. SYXK (Yu) 2013-0002).

Isolation and purification of neurons and astrocytes

The cerebral cortex was isolated in ice-cold D-Hank's solution and separated into 1 mm^3 pieces and digested in Trypsin solution (Amresco, Solon, OH, USA) (37°C, 1.25 mg/mL) for 8 minutes, then medium with 10% fetal bovine serum (FBS; Hyclone, Thermo Scientific, Waltham, MA, USA) was used to suspend the digesting process, and the solution was filtered with 10-mm pore size nylon mesh and centrifuged at 150 × *g* for 4 minutes. The precipitates were re-suspended in DMEM/F12 medium (Invitrogen, Carlsbad, CA, USA) containing 2% B27 (Invitrogen) for neurons and in DMEM/F12 medium containing 10% FBS for astrocytes to a concentration of 1×10^5 cells/mL. Cells were then seeded in plates pre-coated with 0.1 mg/mL poly-L-lysine (molecular weight 70,000–150,000; Hyclone, Thermo Scientific) and cultured at 37°C and 5% CO_2 for 4 hours. Finally, the cells were

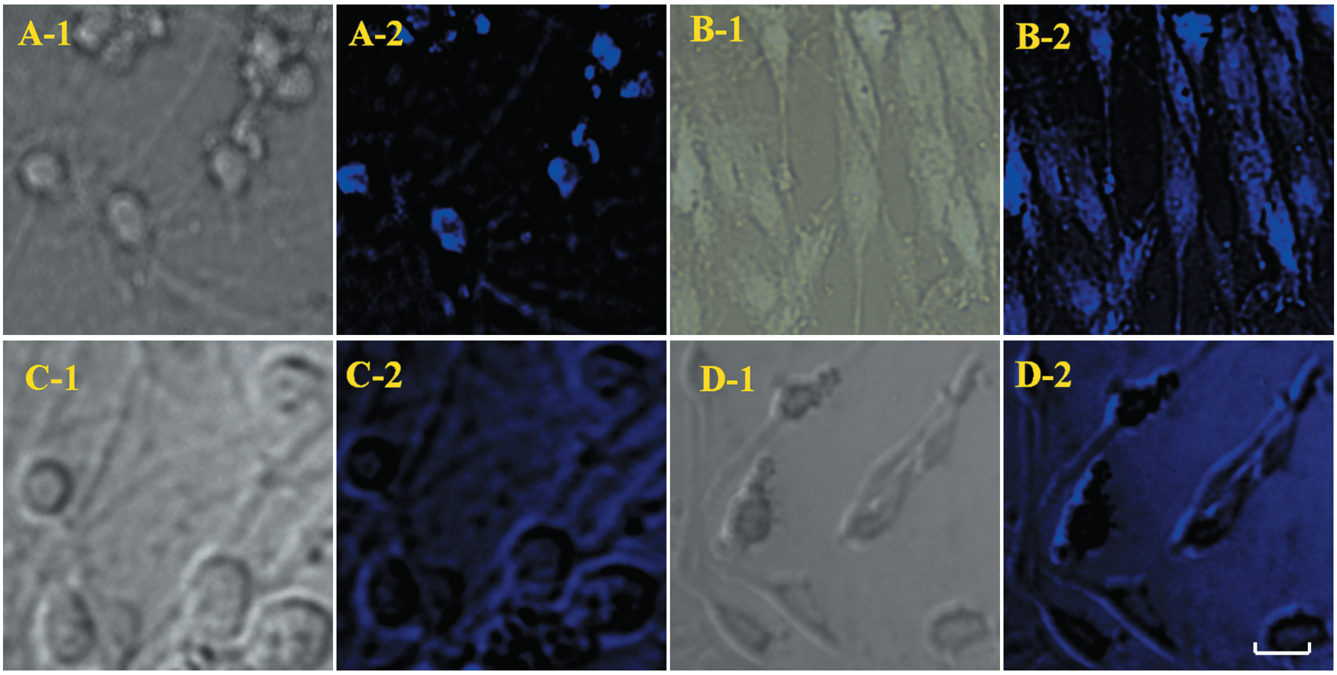

Figure 4 Distribution of puerarin (Pur) in neurons and astrocytes detected by fluorescence microscope.
At 20 minutes after Pur was added, the cell morphologies of neurons (A-1) and astrocytes (B-1) were detected under white light. Blue fluorescence was detected in the neurons (A-2) and astrocytes (B-2), and weak blue fluorescence was detected in the medium (A-2, B-2) under ultraviolet light source. At 60 minutes after Pur was added, the cell morphologies of neurons (C-1) and astrocytes (D-1) were detected under common sources, weak blue fluorescence was detected in the neurons (C-2), and no fluorescence was detected in astrocytes (D-2), but blue fluorescence was detected in the medium (C-2, D-2) under ultraviolet light source. Scale bar: 50 μm.

transferred into new plates and cultured for 24 hours, and the medium was changed every 2 days for 7–10 days. To purify astrocytes, the plate was shaken twice at 220 r/min (37°C, 18 hours).

Immunocytochemical identification of target cells

Briefly, purified cells were fixed with 4% paraformaldehyde at 25°C for 30 minutes, washed with PBS and then incubated in 10% goat serum containing 0.5% Triton X-100 (pH 7.4) for 1 hour. Cells were then probed with different rabbit anti-rat primary antibodies (1:200) overnight at 4°C; microtubule-associated protein-2 (MAP2; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for neurons, and glial fibrillary acidic protein (GFAP; ZSGB-BIO, Beijing, China) for astrocytes. Cells were detected with goat anti-rabbit FITC-IgG (1:800) at 37°C for 1 hour, and the cell nuclei were stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Santa Cruz Biotechnology). Images were captured by fluorescence microscope (Carl Zeiss, Oberkochen, Germany) and the purity of the cells was analyzed by Image-Pro Plus 6.0 (Media Cybernetics, MD, USA).

Co-culture of neurons and astrocytes

The method used to co-culture the two kinds of cells was carried out as previously described but with some modifications (Xue et al., 2013). As shown in **Figure 1**, astrocytes (1.5×10^5 cells/cm^2) were seeded on the outside of a Transwell membrane (Corning, Corning, NY, USA) coated with poly-L-lysine. After culturing cells for 6 hours and subsequently washing with D-Hank's solution, the Transwell was inserted into a plate well containing neurons that had already had been seeded at a density of 1×10^5 cells/cm^2 and subsequently grown for 10 days. DMEM/F12 (1:1) complete medium with 10% FBS and 2% B27 was used to culture cells for 3 days, and the medium was given a daily change before analysis.

Preparation of cell extracts

Pur was added into the medium of co-cultured or cultured neurons and astrocytes at a final concentration of 1,000 ng/mL. At 0, 10, 20, 30, 60, and 90 minutes after the drug was added, the cells were washed with 0.01 M PBS (twice for 30 seconds) and digested with trypsin (1.25 mg/mL) separately and re-suspended in PBS at a concentration of 5×10^5 cells/mL into a final volume of 2 mL. Cells were completely broken using ultrasonication, and after centrifugation at $1.5 \times 10^4 \times g$ for 20 minutes, the supernatant was collected as cell extracts and detected by a fluorospectrophotometer (Hitachi, Tokyo, Japan).

Analytical method

Neuron and astrocyte cell extracts were obtained at a rate of 1:1. 50 ng/mL Pur was prepared with the cell extract to detect the most intensive excitation and emission wavelengths, and both the excitation and the emission slits were 10 nm (Chauhan et al., 2011). Samples of 6.25, 12.5, 25, 50, and 100 ng/mL of Pur prepared with the cell extract were used to fit the standard curve of fluorescence spectrophotometry, and samples of 6.25, 25, and 100 ng/mL Pur were prepared to detect the precision and the percentage recovery; cell extracts without Pur was used to detect the specificity of the method.

Table 1 Validation of the fluorescence spectrophotometry method

Actual concentration (ng/mL)	Precision				Determined concentration (ng/mL)	Recovery (%)
	Intra-day (ng/mL)	RSD (%)	Inter-day (ng/mL)	RSD (%)		
6.25	6.29±0.10	1.58	6.17±0.12	2.94	6.06±0.12	96.96±0.02
25	24.84±0.09	0.36	24.70±0.48	1.92	24.71±0.21	98.84±0.02
100	99.53±0.43	0.83	98.73±1.79	1.81	99.22±0.36	99.19±0.01

The detections were repeated five times. The data were expressed as the mean ± SD except for relative standard deviation (RSD).

Table 2 Comparison of the proposed fluorescence spectrophotometry method with other methods for determination of puerarin

Analytical method	Samples	Working range (μg/mL)	Analysis time (minute)	Limit of detection (ng/mL)	Recovery (%)	Reference
Fluorescence spectrophotometry	*Pueraria tuberosa*	0.01–1.00	1.00	38.62	99.74–100.07	Chauhan et al. (2011)
HPLC	Kampo medicine	0.005–0.50	15.20	3.00	102.00–106.00	Okamura et al. (1999)
UPLC-MS/MS	Rat plasma	0.018–3.72	36.00	3.72	96.17–100.97	Wang et al. (2011)
Capillary zone electrophoresis	*Xinkeshu* capsules and biological samples	17.30–138.00	6.30	34.60	97.70–103.00	Liu et al. (2011)
Flow-injection chemiluminescence	Urine	0.08–10.00	0.50	50.00	91.40–104.80	Yang et al. (2011)
Fluorescence spectrophotometry	Cells	0.006–0.10	1.00	5.45	96.96–99.19	This work

HPLC: High-performance liquid chromatography; UPLC-MS/MS: ultra high performance liquid chromatography-tandem mass spectrometry.

Fluorescence spectrophotometry and fluorescence microscope detection

At 0, 10, 20, 30, 60, and 90 minutes after 1,000 ng/mL Pur was added into the medium, the concentration of Pur in cells was detected through the use of the spectrofluorometer and determined from the standard curve. For fluorescence microscope detection, 20 and 60 minutes after the addition of Pur, neurons and astrocytes were separately washed in DMEM/F12 medium twice and then immediately detected under blue emission of a fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

Statistical analysis

The mean, standard deviation, and relative standard deviation of the concentration of Pur in cells were analyzed by SPSS 19.0 (IBM, Armonk, NY, USA). A value of $P < 0.05$ was considered significantly different.

Results

Identification of neurons and astrocytes

After detection by anti-MAP2 and anti-GFAP, the purities of neurons and astrocytes were 96.50 ± 0.87% and 97.67 ± 0.10%, respectively ($n = 6$).

Establishment and validation of the analytical method

As shown in **Figure 2**, the λex/em of Pur was 342/461 nm, the slit of ex/em was 10 nm, the standard curve exhibited a good linear relationship between absorbance and Pur concentration in the tested concentrations ($y = 4.5107x–0.375$, $R^2 = 0.9978$), and the limit of detection was 5.45 ng/mL. The specificity, precision, and percentage recovery of the method were shown in **Figure 2** and **Table 1**.

Fluorescence spectrophotometry detection for the concentration of Pur in cells

Different distributions and retention time of Pur in tested cells are shown in **Figure 3**. Briefly, peak concentration of Pur in separately cultured or co-cultured neurons was higher than that observed in astrocytes, in particular the concentration of Pur in both co-cultured and separately cultured neurons were kept at a steady level of approximately 12.80 ng/mL, but a scant amount of Pur was detected in astrocytes when co-cultured with Pur for 60–90 minutes.

Fluorescence microscope detection for the distribution of Pur in cells

As shown in **Figure 4**, when cells were co-cultured with Pur for 20 minutes, blue fluorescence was detected in both neurons (**Figure 4A-2**) and astrocytes (**Figure 4B-2**), and a scant amount of blue fluorescence was detected in the cell medium (**Figure 4A-2**, **B-2**). When co-cultured with Pur for 60 minutes, blue fluorescence was detected in the medium (**Figure 4C-2**, **D-2**), weak fluorescence was detected in the neurons (**Figure 4C-2**), but a scant amount of fluorescence was detected in astrocytes (**Figure 4D-2**).

Discussion

The injection of Pur has been widely used in clinical therapy of ischemic cerebrovascular disease, coronary heart disease, angina pectoris, myocardial infarction, and diabetes in China, but its beneficial outcomes on human health are still unknown (Wang et al., 2006; Tan et al., 2008; Wei et al., 2014). Correctly assessing cytoprotection of Pur remains a major challenge and target cells in different organs should be confirmed. The plasma pharmacokinetics of Pur in rats were widely researched (Okamura et al., 1999; Li et al., 2013), but were limited by the difficulties in detecting the concentration of drugs in each kind of neurocyte in brain tissue; to the best of our knowledge, there have been no studies addressing the distribution of Pur in different kinds of neurocytes until now. In this study, a system for co-culturing neurons and astrocytes was established and first used to analyze the distribution and retention time of Pur in neurons and astrocytes. This offered a new method for detecting the distribution of drugs in different neurocytes. Results showed that there was a broader distribution and longer retention time of Pur in neurons than in astrocytes, which might provide some information towards understanding the neuroprotective mechanism of this significant natural medicine.

Several methods have been used for the detection of Pur in different sample matrices, such as high-performance liquid chromatography (Okamura et al., 1999), ultra-performance liquid chromatography tandem mass-spectrometry (Li et al., 2013), capillary zone electrophoresis (Liu et al., 2012), and flow-injection chemiluminescence (Yang et al., 2011), but all these methods are time-consuming and costly. Based on the isoflavone structure, Pur showed intense blue fluorescence observed under UV light, so the content of Pur in Pueraria tuberosa could be detected by a spectrofluorometric method (Chauhan et al., 2011). However, there is no report on this method being used to detect Pur in cells, and so a rapid and simple fluorometric assay method for the measurement of Pur concentration in cells (which had been previously confirmed to have acceptable linearity, precision, and recovery), was established in this study and used to detect the distribution of Pur in co-culture and separately cultured neurons and astrocytes. **Table 2** shows Pur distribution in different kinds of samples detected by different methods. Results from this study showed that when co-cultured with Pur for 60–90 minutes, there was a scant amount of Pur in the tested astrocytes, but the concentration of Pur in both separately cultured and co-cultured neurons was maintained at a relatively stable level, and the underlying mechanism should be further investigated.

Author contributions: *SYW, BBF and XYX designed the study. SYW, JT, QX and FHS performed the experiments. SYW, YJL and YL analyzed the data. SYW and XYX wrote the paper. All authors approved the final version of this paper.*
Conflicts of interest: *None declared.*

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