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Effects of puerarin against glutamate excitotoxicity on cultured mouse cerebral cortical neurons

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KEY WORDS puerarin; neurons; sodium glutamate; *N*-methylaspartate; kainic acid; kynurenic acid; quinoxalines; lactate dehydrogenase

AIM: To study the effects of puerarin (Pue) against injury of cultured neurons by sodium glutamate (Glu). **METHODS**: Neuronal damage induced by Glu, *N*-methyl-*D*-aspartate (NMDA), and kainic acid (KA), as well as the actions of Pue and some excitatory amino acid antagonists (EAAA), were measured by determining the leakage of lactate dehydrogenase (LDH) from nerve cells. **RESULTS**: The 24-h leakage of LDH was increased from cells exposed either to Glu 100 and 500 $\mu mol \cdot L^{-1}$ for 15 min (from 20 ± 4 kU/g protein in control group to 35 ± 3 kU/g protein in Glu 100 $\mu mol \cdot L^{-1}$ group and to 46 ± 6 kU/g protein in Glu 500 $\mu mol \cdot L^{-1}$ group) or to NMDA 500 $\mu mol \cdot L^{-1}$ or KA 500 $\mu mol \cdot L^{-1}$ for 45 min (from 19 ± 4 kU/g protein in control group to 27 ± 3 kU/g protein in NMDA group and to 30 ± 5 kU/g protein in KA group). Pre and post-treatment with Pue (100 $\mu mol \cdot L^{-1}$) decreased the leakage of LDH, which was similar to the effects of EAAA kynurenic acid (from 35 ± 3 kU/g protein in Glu 100 $\mu mol \cdot L^{-1}$ to 20 ± 5 kU/g protein in kynurenic acid group and to 22 ± 3 kU/g protein in Pue group), *DL*-2-amino-5-phosphonovaleric acid (APV) (from 27 ± 3 kU/g protein in NMDA damaged group to 183 kU/g protein in APV group and to 19 ± 5 kU/g protein in Pue group) or 6, 7-dinitroquinoxaline-2,3(1H,4H)-diane (DNQX) (from 30 ± 5 kU/g protein in KA damaged control to 22 ± 5 kU/g protein in DNQX group and to 20 ± 4 kU/g protein in Pue group). Post-treatment with Pue (100 $\mu mol \cdot L^{-1}$) was able to reduce 24-h leakage of LDH from neurons expos ed to Glu 100 $\mu mol \cdot L^{-1}$ for 15 min (from 35 ± 3 kU/g protein to 27 ± 4 kU/g protein). **CONCLUSION**: Pue had protective effects on neurons damaged by Glu, NMDA, or KA.

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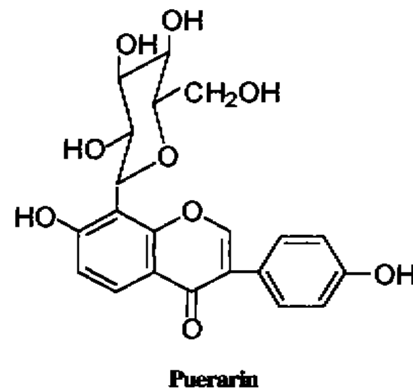
Glutamate (Glu) is an excitatory neurotransmitter in the mammalian brain. In some neuropathothies, such as cerebral hypoxia-ischemia, epilepsy, Huntington's disease, and Alzheimer's disease, excessive release, abnormal leakage, and impaired uptake of Glu result in extracellular Glu accumulation. Glu causes neuronal damage mainly via *N*-methyl-*D*-aspartic acid (NMDA) and kainic acid (KA) receptors[1]. Many Glu receptor antagonists have protective effects on neurons, but their effects are not satisfactory in the clinic. Work was done in many countries to search for low toxic and non-specific drugs with protective effects on brain. Puerarin (Pue), 8-C-C-glucopyranosy 1-4′-7-dihydro xyisoflavone, has been used for the treatment of ischemic and reperfusion injury to the heart[2-4] and arterial obstruction of retina[5]. We found it affected the function of neurons via the inhibition of a subtype of sodium channels[6]. It would be intriguing to know whether Pue protects the brain against Glu neurotoxicity. The present study was to investigate whether Pue could prevent EAA-induced damage in cultured mouse cerebral cortical neurons.



Puerarin

MATERIALS AND METHODS

Chemicals Puerarin (Pue) was supplied

by the Institute of Materia Medica, Chinese Academy of Medical Sciences (purity > 95 %). *L*-glutamic acid monosodium salt (Glu) was obtained from Shanghai Institute of Biochemistry, Chinese Academy of Sciences. Glycine was obtained from Beijing Chemical Factory. *N*-methyl-*D*-aspartic acid (NMDA), kainic acid (KA), kynurenic acid, *DL*-2-amino-5-phosphonovaleric acid (APV), and 6,7-dinitroquinoxaline-2,3(1H,4H)-diane (DNQX) were bought from Sigma Co.

The chemicals were dissolved in Mg^{2+}-free balanced salt solution (BSS): NaCl 135, KCl 5, $CaCl_2$ 1.8, $NaHCO_3$ 3.6, glucose 7.5, HEPES 10 $mmol \cdot L^{-1}$, pH 7.4.

Cell cultures Primary neuron cultures were prepared following the described procedure[7].

Protocol The 9 – 11-d cultures were washed twice with BSS. The cells were incubated with Glu or NMDA or KA at 37 ℃. To increase the response of EAA receptor to Glu, glycine equalling to 1/5 Glu concentration was added to the groups exposed to Glu[8]. The cultures treated with BSS served as control. After Glu, NMDA or KA (EAA) exposure, the cells were washed twice with MEM, returned to the initial culture medium and maintained in a CO_2 incubator for 24 h. Cultures were divided into 5 groups (Fig 1).

Group A) Glu damage group: the cultures were exposed to Glu 50, 100, or 500 $\mu mol \cdot L^{-1}$ for 15 min.

Group B) Glu + chemicals: there were 2 subgroups: a) Pre- and post-treatment: Pue (1, 10, and 100 $\mu mol \cdot L^{-1}$) or kynurenic acid (100 $\mu mol \cdot L^{-1}$) given 1 h before and during the 15-min exposure to Glu 100 $\mu mol \cdot L^{-1}$ and for the following 24 h. b) Post-treatment: Pue (10 or 100 $\mu mol \cdot L^{-1}$) was added to the cultures for 24 h following the 15-min exposure to Glu 100 $\mu mol \cdot L^{-1}$.

Group C) NMDA damage and pre- and post-treatment with chemicals: the cultures exposed to NMDA 500 $\mu mol \cdot L^{-1}$ for 45 min served as damage group. Pre- and post-treatment with chemicals meant Pue 100 $\mu mol \cdot L^{-1}$ or APV 100 $\mu mol \cdot L^{-1}$ administered 1 h before and during the 45 min exposure to NMDA 500 $\mu mol \cdot L^{-1}$ and for the following 24 h.

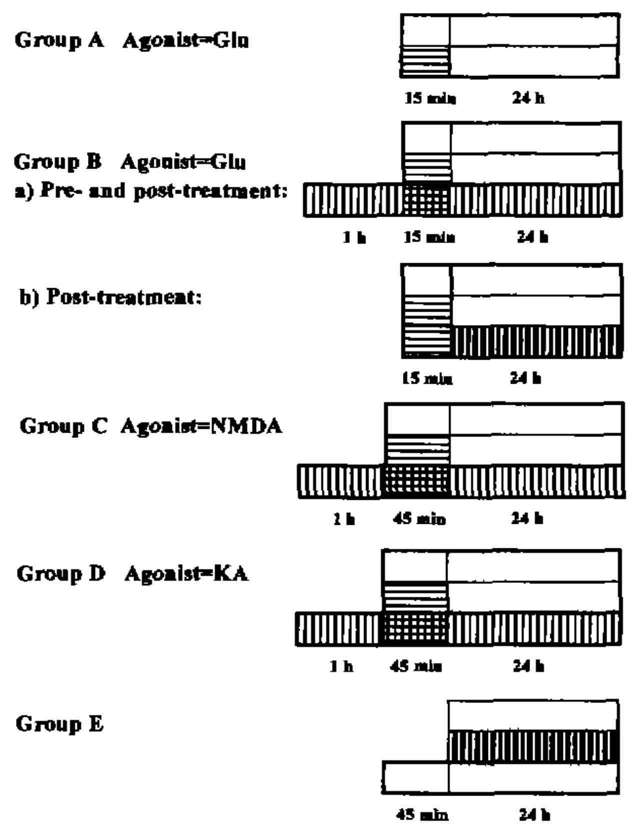


Fig 1. Experimental protocals. Mg²⁺-free BSS, MEM, agonist, chemicals: Pue or antagonists (kynurenic acid in group B; APV in group C; DNQX in group D), Pue.

Group D) KA damage and pre- and post-treatment with chemicals: the cultures exposed to KA 500 $\mu mol \cdot L^{-1}$ for 45 min served as damage group. Pre- and post-treatment with chemicals meant Pue 100 $\mu mol \cdot L^{-1}$ or DNQX 100 $\mu mol \cdot L^{-1}$ administered 1 h before and during the 45 min exposure to KA 500 $\mu mol \cdot L^{-1}$ and for the following 24 h.

Group E) Pue or BSS treatment: The cultures were treated with Pue 100 $\mu mol \cdot L^{-1}$ for 24 h or BSS for 45 min.

Determination of LDH and protein Following the 24-h incubation, the LDH activities in medium were determined using the Kit for LDH (Beijing Chemical Factory). The cells were digested in NaOH 0.2 $mol \cdot L^{-1}$ for protein

determination[9]. LDH activities were expressed as kU/g protein.

Statistics Results were presented as $\bar{x} \pm s$ and analyzed by t test.

RESULTS

Glu injury Exposure to Glu 100 $\mu mol \cdot L^{-1}$ for 15 min was sufficient to induce neuronal damage by the next day, as evidenced by leakage of LDH (Tab 1).

Pue against Glu injury Pre- and post-treatment with Pue 1 – 100 $\mu mol \cdot L^{-1}$ concentration-dependently reduced the LDH leakage induced by Glu 100 $\mu mol \cdot L^{-1}$. Pue 100 $\mu mol \cdot L^{-1}$ decreased LDH leakage from 35 ± 3 kU/g protein to 22 ± 3 kU/g protein ($P < 0.01$). The potency was similar to that of kynurenic acid 100 $\mu mol \cdot L^{-1}$ (Tab 1). Post-treatment with Pue 100 $\mu mol \cdot L^{-1}$ also attenuated neuron damage caused by Glu. The leakage of LDH was lowered from 35 ± 3 kU/g protein to 27 ± 4 kU/g protein (Tab 1).

Pue against NMDA and KA injury In cultures treated with NMDA or KA 500 $\mu mol \cdot L^{-1}$, the LDH leakages were increased (27 ± 3 kU/g protein or 30 ± 5 kU/g protein respectively, $P < 0.01$ *vs* control). Pre- and post-treatment with Pue 100 $\mu mol \cdot L^{-1}$ remarkably reduced the leakage of LDH induced by NMDA or KA, resembling the effects of APV or DNQX, respectively (Tab 1).

When cultures were treated with Pue 100 $\mu mol \cdot L^{-1}$ for 24 h or BSS for 45 min, the 24-h leakage of LDH from the cells did not change (Tab 1).

DISCUSSION

When EAA receptors are excited, Na^{+} and Ca^{2+} influx increase, accompanied by passive Cl^{-} and water influx[10]. These induce the neuronal swelling and the leakage of LDH. An excessive Ca^{2+} influx and resultant cellular Ca^{2+} overload lead to generation of free radicals. Once free radicals are formed, they may promote further excitotoxic injury by promoting glutamate release[11].

Exposure of cultured neurons to toxic glutamate concentration for several minutes

Tab 1. The 24-h leakage of LDH from neurons after Glu, NMDA, or KA exposure and effect of treatment with chemicals on 24-h leakage of LDH from neurons exposed to Glu, NMDA, or KA. n = experiments from 3 – 6 rats, $\bar{x} \pm s$.
$^{a}P > 0.05$ *vs* MEM group. $^{d}P > 0.05$, $^{f}P < 0.01$ *vs* BSS control A. $^{g}P > 0.05$, $^{i}P < 0.01$ *vs* Glu 100 $\mu mol \cdot L^{-1}$ group. $^{l}P < 0.01$ *vs* BBS control B. $^{o}P < 0.01$ *vs* NMDA group. $^{r}P < 0.01$ *vs* KA group.

Exposure, $\mu mol \cdot L^{-1}$	Chemicals, $\mu mol \cdot L^{-1}$ Pre-treatment	Post-treatment	n	LDH leakage, kU/g protein
MEM (– , 24 h)	–	–	6	20 ± 1
Pue (100, 24 h)	–	–	5	21 ± 5^{a}
BSS control A (– , 15 min)	–	–	12	20 ± 4^{a}
Glu (50, 15 min)	–	–	10	26 ± 11^{d}
Glu (100, 15 min)	–	–	12	35 ± 3^{f}
Glu (500, 15 min)	–	–	6	46 ± 6^{f}
Glu (100, 15 min)	Kynurenic acid (100)	Kynurenic acid (100)	6	20 ± 5^{i}
Glu (100, 15 min)	Pue (1)	Pue (1)	6	34 ± 2^{g}
Glu (100, 15 min)	Pue (10)	Pue (10)	6	28 ± 6^{i}
Glu (100, 15 min)	Pue (100)	Pue (100)	5	22 ± 3^{i}
Glu (100, 15 min)	–	Pue (10)	7	32 ± 3^{g}
Glu (100, 15 min)	–	Pue (100)	12	27 ± 4^{i}
BSS control B (– , 45 min)	–	–	11	19 ± 4^{a}
NMDA (500, 45 min)	–	–	5	27 ± 3^{l}
NMDA (500, 45 min)	APV (100)	APV (100)	6	18 ± 3°
NMDA (500, 45 min)	Pue (100)	Pue (100)	6	19 ± 5°
KA (500, 45 min)	–	–	6	30 ± 5^{l}
KA (500, 45 min)	DNQX (100)	DNQX (100)	6	22 ± 5^{r}
KA (500, 45 min)	Pue (100)	Pu e (100)	5	20 ± 4^{r}

results in neurotoxicity which develops in the following 24 h. This is an experimental paradigm used commonly to study the delayed neurotoxicity of Glu[12,13]. In our experiments, a short exposure of NMDA or KA 500 $\mu mol \cdot L^{-1}$, or Glu 100 $\mu mol \cdot L^{-1}$, produced damage of neuronal cells, which was in agreement with previous reports[13,14].

The present study demonstrated that Pue in the concentration range of 10 – 100 $\mu mol \cdot L^{-1}$ attenuated the damage induced by Glu, NMDA, or KA on mouse cerebral neurons, as evidenced by a decreasing leakage of LDH. The protective effects resembled the effects of kynurenic acid, AP-5 or DNQX. Because Pue had protective effects on myocardium[3,4] and retina[5], we conjecture that the protective effects of Pue on cells may have some mechanisms in common. One possible mechanism is against free radicals damage. It has been found that Pue protected myocardial reperfusion injury[3,4]. Another possible mechanism is inhibition of Pue on ion influx through cell membrane. It was reported that Pue had antidysrhythmic effect[2] and inhibited sodium current in rat dorsal root ganglion neurons[6].

Our results demonstrated that Pue had protective effects on nerve cells against the injury of Glu at cellular level.

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葛根素抗谷氨酸对小鼠神经细胞兴奋毒的作用

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关键词 葛根素；神经元；谷氨酸钠；*N*-甲基天冬氨酸；卡因酸；犬尿烯酸；喹噁啉类；乳酸脱氢酶

目的：研究葛根素(Pue)对谷氨酸钠(Glu)引起的神经细胞损伤的作用．**方法**：分光光度法测定神经细胞乳酸脱氢酶(LDH)的漏出．**结果**：Glu 100 或 500 $\mu mol \cdot L^{-1}$作用于神经细胞 15 min，*N*-甲基-D-天冬氨酸(NMDA)或卡因酸(KA) 500 $\mu mol \cdot L^{-1}$作用于神经细胞 45 min，24 h后 LDH 漏出明显增加；当细胞被 Glu、NMDA 或 KA 损伤前 1 h、同时和之后的24 h加入 Pue 100 $\mu mol \cdot L^{-1}$，能明显减少 LDH 的漏出，其作用与 EAA 拮抗剂犬尿烯酸、2-氨基-5-磷酸基戊酸(APV)和喹噁啉类(DNQX)相似．在谷氨酸作用于神经细胞 15 min 后，加入 Pue 100 $\mu mol \cdot L^{-1}$作用24 h，也能减少 LDH 的漏出．**结论**：Pue 对 Glu、NMDA 或 KA 损伤的神经细胞有保护作用．