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Effect of puerarin on glutamine synthetase activity in rat retina following acute intraocular hypertension

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This study was conducted to demonstrate whether puerarin regulates glutamine synthetase (GS) activity following intraocular hypertension and has therapeutic potential in ophthalmology for the protection of optic nerves in patients with glaucoma. This study used a Wistar rat model of acute closed-angle glaucoma to investigate the effect of puerarin on GS activity in rat retina following intraocular hypertension. Acute intraocular hypertension was induced by increasing anterior chamber pressure to 110 mmHg for 30 min in the left eyes of 50 Wistar rats, while 5 additional Wistar rats lacking intraocular hypertension were used as a control group. Retinal GS activity was measured at 4, 12, 24, 36 and 72 h after induction of acute intraocular hypertension with/without puerarin treatment. Compared to the control group that lacked intraocular hypertension, GS activity in the intraocular hypertension group significantly decreased at 4 and 12 h (P<0.01), before increasing at 24 to 36 h and restoring to a level similar to the control group at 72 h. However, puerarin significantly (P<0.05) prevented the loss of GS activity seen in the intraocular hypertension group at 4 and 12 h, with no significant (P<0.05) difference in GS activity noted between the control group and rats treated with puerarin at these early time points. GS activity significantly (P<0.05) increased above control values at 24 and 36 h in the puerarin-treated group before eventually restoring to control levels at 72 h. These findings suggest puerarin protects GS activity in the early stages of retinal acute intraocular hypertension and may be of potential therapeutic benefit in acute closed-angle glaucoma.

Key words: Puerarin, acute intraocular hypertension, glutathione synthetase.

INTRODUCTION

Puerarin is one of several isoflavones found in a number of plants and herbs in East Asia, including the root of Pueraria, notably the leguminous kudzu plant (*Radix puerariae*) (Yeung et al., 2006). Puerarin (4, 7- dihydroxy -8-β-D glucose isoflavone) is typically purified from dried root extracts of kudzu. Puerarin has been shown to dilate coronary and cerebral vascular smooth muscle, reduce vascular resistance and myocardial oxygen consumption,

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improve myocardial contractility and microcirculation function, and inhibit platelet aggregation (Tam et al., 2009). Puerarin has been used in clinical practice to treat human diseases including ischemic heart disease and cerebrovascular/retinal diseases (Zhang et al., 2018; Liu et al., 2018). In the treatment of retinal diseases, puerarin crosses the blood-ocular barrier into the aqueous, vitreous region of the eye (Wong et al., 2011; Song et al., 2020). Mechanisms of action reported for puerarin include inhibition of adenylate cyclase, stimulation of antioxidant activity (Zhao et al., 2015; Zhou et al., 2014; Xu et al., 2016), induction of superoxide dismutase activity, enhancement of p450 activity, improvement in ocular microcirculation, inhibition of Ca^{2+} influx through calcium channels (Song et al., 2020; Li et al., 2017) and enhancement of anti-apoptotic activities (Li et al., 2008; Yu et al., 2009; He et al., 2009). Puerarin has also been promoted as a therapeutic strategy for diabetic nephropathy by reducing serum levels of tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) (Xu et al., 2016; Wang et al., 2014). Furthermore, puerarin acts as an anti-inflammatory agent by blocking nuclear factor-kappa beta (NF–kB) signalling, suggesting it may be a useful prophylactic therapy for the prevention of atherosclerosis (Yang et al., 2010). Glutamine synthetase (GS) is the key enzyme in the glutamic acid-glutamine cycle and it plays an important role in protecting the optic nerves by reducing the extracellular concentration of glutamic acid (glutamate). Glutamate is a major excitatory neurotransmitter that participates in neuron signalling by activating a variety of ionotropic and metabotropic glutamate receptors in the mammalian central nervous system and retina. Glutamate-induced excitotoxicity has been proposed to mediate the death of retinal ganglion cells in glaucoma (Moreno et al., 2005).

Furthermore, altered GS activity has been found in many kinds of human diseases, including diabetic retinal disease, epilepsy and ischemic optic nerve disease (Moreno et al., 2005; Sáenz et al., 2004). Under normal physiological circumstances, retinal extracellular glutamate is taken up by GS-expressing Müller cells that carry out the transformation of glutamate to glutamine (Zhang et al., 2014).

However, conditions that reduce GS levels or activity would decrease this glutamate metabolism and might result in detrimental increases in extracellular glutamate concentration. Thus, this study investigated the effect of puerarin on GS activity during acute intraocular hypertension in a Wister rat model of acute closed-angle glaucoma to assess whether puerarin could provide retinal/optic nerve protection. Puerarin reversed the inhibition of GS activity induced by acute high intracellular pressure, suggesting puerarin has therapeutic potential in ophthalmology for the protection of optic nerves in the treatment of glaucoma. The precise mechanism by which puerarin provides this protection against acute high intraocular pressure in the rat retina warrants further investigation.

MATERIALS AND METHODS

Animals

Ethical approval

All animal studies and experimental procedures were approved by the Ethical Committee at Weifang People's Hospital, Weifang, China. Healthy, eight-week-old Wistar rats were supplied by the Animal Research Centre, Weifang Medical University, Weifang, China. The Wistar rats were kept in ventilated cages and maintained in a temperature-controlled room with a 12:12 h light:dark schedule and provided with free access to water and standard rat pelleted diet *ad libitum*.

Puerarin treatment and retinal tissue preparation

A total of 55 pure-bred healthy Wistar rats (200-250 g) of either sex were divided randomly into 11 units (5 rats per unit) and allocated to the following three groups: control group (1 unit) that had healthy/normal characteristics and lacked the application of intraocular hypertension; intraocular hypertension group (5 units) lacking puerarin treatment and consisting of one unit (5 rats) per experimental time point (4, 12, 24, 36 and 72 h); and puerarin-treated intraocular hypertension group (5 units) again consisting of one unit (5 rats) per experimental time point (4, 12, 24, 36 and 72 h). Experiments were conducted in accordance with standard approved laboratory conditions for animal experiments. No eye diseases were present in any of the animals, and the left eyes were used throughout the experiment. The acute high intraocular pressure model was generated by left eye anterior chamber pressure perfusion. The rats were anaesthetised by intraperitoneal injection of 0.8 ml of 10% chloral hydrate. Puerarin (100 mg/kg, Enbei Pharmaceutical Co. Ltd, Zhejiang, China) was administered to the puerarin-treated intraocular hypertension group by slow intravenous injection into each rat's tail 30 min before applying intraocular pressure. A similar sham intravenous injection was applied to the control and intraocular hypertension groups but no puerarin was administered. To induce high intraocular hypertension, an acupuncture needle was inserted into the left eye anterior chamber, and saline was infused for 30 min using a saline infusion bottle set to 150 cm (corresponding to a pressure of 110 mmHg). In the sham-treated control group, an acupuncture needle was inserted into the left eye anterior chamber and left in place for 30 min without infusing any saline. All the rats were then euthanised using excess chloral hydrate at 4, 12, 24, 36 or 72 h after intraocular pressure was applied. The eyes were removed quickly, the cornea cut out and the crystal vitreous removed. The retina was carefully peeled off and placed into liquid nitrogen immediately.

Protein extraction

Rat retinal tissue was placed into a mortar with some liquid nitrogen, and a pestle used to slowly grind the tissue. The tissue was then placed in ice-cold homogenate lysis buffer (Tris-hydrochloride (Tris-HCl), NP-40, sodium chloride (NaCl), ethylenediaminetetraacetic acid (EDTA), sodium azide (NaN_3) and PMSF protease inhibitor at pH 7.5) and homogenised for 10 cycles on ice (each cycle consisted of vortex mixing followed by sonication of the tissue homogenate for 2 min). Subsequently, the samples were centrifuged at 10,000×g for 10 min at 4°C prior to collecting supernatants and storing -20°C.

Protein assay

The concentrations of protein were measured using the Bio-Rad protein assay according to the method described by the manufacturer (Bio-Rad). The concentration of total protein in samples was set to 1 mg/ml in all cases.

GS detection

The GS detection kit was supplied by Jiancheng Biological Engineering Institute, Nanjing, China. The kit was based on a colorimetric reaction between glutamine and hydroxylamine which can generate T-glutamyl hydroxamate and ammonia in the presence of GS. The latter was measured by its absorbance (Ab) at 595 nm and GS activity in each sample was determined according to the manufacturer's protocol using the equation:

GS activity (U/mg) = (sample Ab - blank Ab)/(standard Ab - blank Ab) × standard concentration (20 µM/ml) × (4 mg/ml).

Statistical analysis

The data were analysed using SPSS 11.0 software and presented as mean ± standard deviation (SD) for at least three experiments. Univariate analysis of variance was used to detect the overall statistical difference between the groups as a whole. Dunnett's t-test for comparison of two groups was subsequently applied to make pair-wise comparisons between the experimental groups. A P-value of less than 0.05 was considered statistically significant in all analyses.

RESULTS AND DISCUSSION

In this study, the influence of puerarin on GS activity after acute high intraocular pressure was investigated. The study utilised an established acute high eye pressure model in rats to explore differences in GS expression with time. GS activity (U/mg of protein; mean ± SD, n = 5) was monitored at 4, 12, 24, 36 and 72 h after left eye acute intraocular high-pressure application, with and without the puerarin injection. GS activity was down-regulated after acute intraocular hypertension in the left eyes at 4 and 12 h (P<0.01) compared to the normal control (Table 1 and Figure 1), albeit an increase between 4 and 12 h. GS activity in the high intraocular hypertension group recovered to a similar level to that in the control group (P>0.05) at 24 h.

Interestingly, GS activity became significantly greater in the high intraocular hypertension group than in the control group (P<0.01) at 36 h, before returning to similar levels as the control group at 72 h.

Puerarin treatment reversed the inhibitory effects of high intraocular hypertension on GS activity at 4 and 12 h, with no significant differences in GS activity detected at 4 and 12 h compared to the control group. Indeed, GS activity was significantly (P<0.01) higher in the puerarin-treated group compared to the non-puerarin treated high intraocular hypertension group at 4, 12 and 24 h. GS activity became significantly greater in the puerarin group than the control group (P<0.01) at 24 and 36 h, peaking at 36 h before returning to similar levels as the control group at 72 h.

The results suggest acute high intraocular pressure initiates a self-protection response in rats, whereby GS activity is subsequently restored after an initial period of inhibition. These findings are consistent with those reported in the literature. Early (<24 h) inhibition followed by significant elevation of GS activity that gradually returned to normal levels has been previously demonstrated in rat models of high intraocular pressure (Gui et al., 2007; Shen et al., 2004). Elevated intraocular pressure has been shown to suppress early activity and expression of GS in an *ex vivo* rat model, leading to glutamate-associated excitotoxicity in rat retinal ganglion cells (Ishikawa et al., 2011). However, this study has shown puerarin treatment can reverse the early (<24 hours) inhibition of GS activity, suggesting it may limit retinal injury caused by early acute intraocular high pressure. The stimulation of GS activity by puerarin is likely to protect retinal ganglion cells from glutamate-mediated damage and/or apoptosis by promoting the conversion of glutamate into glutamine. Indeed, several studies have shown that excessive glutamate is an important mechanism in glaucoma optic nerve damage (Ishikawa et al., 2011; Otori et al., 1998; Asai et al., 2000). However, although an increase in GS activity may be indicative of increased enzyme levels and subsequent substrate turnover; future work will need to confirm whether puerarin acts by inducing GS gene transcription or via some other mechanism. Interestingly, high intraocular pressure for 24 h has been shown to inhibit the expression of a key glutamate transporter in the retina called glutamate aspartate transporter (GLAST) and it is this impairment of GLAST that likely results in the downregulation of GS activity (Ishikawa et al., 2011). Thus, it feasible puerarin may indirectly increase early GS activity following high intraocular pressure through its action on an intermediary such as GLAST.

Limitations of the study

(1) This study has shown that puerarin protects GS activity in a rat model of retinal acute intraocular hypertension. However, the precise mechanism of action by which puerarin achieves this has not been determined. Thus, future work will focus on molecular investigations to elucidate these underlying mechanisms, together with histopathological (e.g. retinal ganglion cell damage) and biochemical (e.g. glutamate-glutamine) analyses to provide supporting evidence for our initial findings.
(2) This study measured GS activity but did not determine mRNA expression. There was insufficient tissue available within the scope and budget constraints of this preliminary investigation to extract enough high quality protein and RNA from samples. Thus, it was not possible

Table 1. Glutamine synthetase (GS) activity (U/mg protein) in rats with intraocular hypertension with/without puerarin treatment at time T (hours; h).

Group	GS activity (U/mg protein)			Dunnett's t-test	P-value
	Control	Hypertension	Hypertension + Puerarin		
Time T (h)	75.32±4.66				
4	72.06	45.90±5.08$^{\#}$	60.11±4.52	3.45	<0.05
		45.55	60.06		
		51.95	59.41		
		46.68	66.79		
		47.39	54.07		
		37.92	60.22		
12	83.16	56.56±4.74$^{\#}$	73.49±5.12	3.88	<0.05
		53.28	81.75		
		64.92	67.69		
		55.29	72.24		
		54.14	73.42		
		55.18	72.34		
24	73.02	75.91±5.09	87.67±4.78$^{\#}$	3.08	<0.05
		78.05	94.88		
		77.33	87.38		
		78.21	87.56		
		79.10	87.13		
		66.88	81.42		
36	76.02	90.33±5.85$^{\#}$	91.35±5.54$^{\#}$	1.29	>0.05
		93.96	99.67		
		81.48	90.42		
		89.68	91.21		
		96.98	84.10		
		89.56	91.33		
72	72.33	76.12±4.95	77.52±4.76	1.48	>0.05
		79.36	77.72		
		75.19	76.42		
		68.14	71.17		
		77.13	84.51		
		80.79	77.8		

$^{\#}$Indicates significant differences compared to the control group whereas stated P values indicate probability values when comparing the puerarin-treated intraocular hypertension group with the non-puerarin treated intraocular hypertension group.

to confirm whether observed differences in GS activity are a reflection of changes at the level of gene transcription or subsequent protein synthesis/degradation. Future work will determine whether puerarin treatment induces expression of GS mRNA, either directly or via an intermediary following early high intraocular pressure.

(3) The experimental control group consisted of 1 unit (5 rats) rather than 5 units (25 rats). The number of rats used in this healthy control group was actively kept to a minimum (5 rats) to be consistent with the 3 Rs principle (Replacement/Reduction/Refinement) in the animal ethics application, thereby minimizing humane animal usage in the experiments wherever possible. Previous investigations, in addition to the present findings, have shown that GS activity and expression remains relatively unchanged over time in untreated, healthy control sets of rats (Moreno et al., 2005; Shen et al., 2004). Thus, there was limited merit in utilizing another large untreated control group consisting of 25 rats given the consistency in GS activity in this group. In contrast, 5 rats (1 unit) per

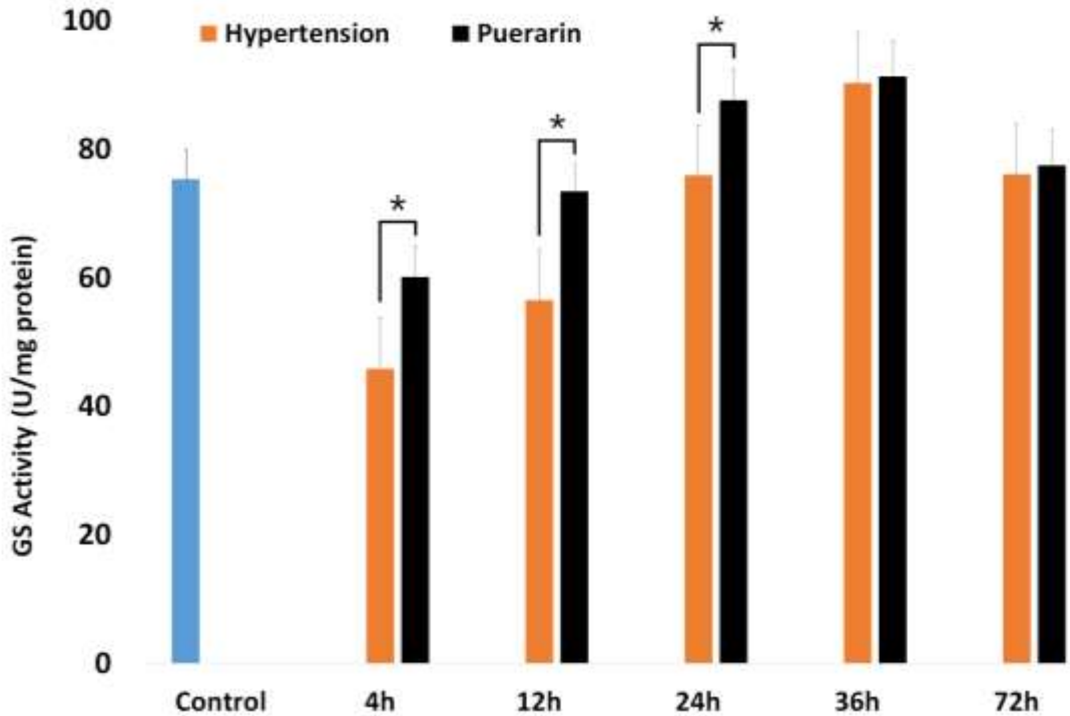

Figure 1. Glutamine synthetase (GS) activity (U/mg of protein) at time T (h) in rats with intraocular hypertension with/without puerarin treatment. *Indicates significant (P<0.05) differences between intraocular hypertension groups treated with or without puerarin. Error bars indicate the standard deviation (SD).

time point were deemed necessary in the other two groups where intraocular hypertension had been applied given this factor was being investigated in our hypothesis and was expected to alter GS activity.

Conclusion

This is the first study to confirm puerarin prevents the early inhibition of GS activity caused by high intraocular hypertension, suggesting it may protect against glutamate-induced excitotoxicity in glaucoma. Indeed, the findings are supported by clinical evidence that puerarin may be effective in the treatment of glaucoma (Kang, 1993; Xu et al., 2010). Further detailed investigations are now required to confirm this hypothesis in other animal models and human studies, together with experiments to interrogate the precise mechanisms of action of puerarin.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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The authors Dr J Zhang and Dr D Liu contributed equally to this work.

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