—Regular Articles—

# Protecting Mechanism of Puerarin on the Brain Neurocyte of Rat in Acute Local Ischemia Brain Injury and Local Cerebral Ischemia-reperfusion Injury

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(Received June 6, 2008; Accepted August 1, 2008)

The present study was designed to investigate the possible properties of the injured brain neurocytes, the expression of heat shock protein70 (HSP70) and Fas protein after acute local ischemia brain injury and local cerebral ischemia-reperfusion injury in rats and to investigate the protecting mechanism of puerarin on the brain neurocytes of rats in acute local ischemia brain injury and local cerebral ischemia-reperfusion injury. A rat model of acute local cerebral ischemia was made by ligatting the middle cerebral artery. The rat model of local cerebral ischemia and reperfusion injury was made by ligatting the middle cerebral artery for 30 min then opened for 30 min. Rats of puerarin treating group were injected with puerarin in dose of 30 mg/kg$^{-1}$ by intraperitoneal injection 30 min before ischemia. HSP70 and Fas protein expressions in brain tissue were detected by SP method of histochemistry. In addition, dead brain neurocytes were counted and their morphology was observed. The results indicated that puerarin can limit the tissue injury caused by local cerebral ischemia injury through improving expression of HSP70, and limit the tissue injury caused by local cerebral ischemia-reperfusion through decreasing the Fas expression and improving expression of HSP70. On the basis of these results, it may be concluded that puerarin can protect the brain neurocytes of rats in acute local ischemia brain injury and local cerebral ischemia-reperfusion injury, which may be different according to the different injury mechanism.

**Key words**——puerarin; cerebral ischemia; cerebral ischemia-reperfusion; heat shock protein70 (HSP70); Fas protein; rat

## INTRODUCTION

Acute cerebral ischemia diseases, leading to multitudinous complications, are common in clinic, and usually cause serious consequences, including staggering economic burden on health care sources and poising to become the leading cause of death and morbidity in the developed and developing countries. Timely restoration of these diseases by thrombolysis[1] and revascularization[2] has been successfully used to rescue some articulo mortis patients. However, reperfusion of cerebral ischemia has its own inherent limitation to produce reperfusion injury.[3,4] Besides, operations have some strict indications and risk.[5–7]

Many pharmacological interventions such as cerebral vasodilator,[8] thrombolytics,[9] $Ca^{2+}$ channel blocker,[10] antioxidant[11] and free radical scavenger[12] have been observed to produce acute ischemia and cerebral ischemia-reperfusion protection. Meanwhile, we still have to face that these medications present various problems such as serious side effects, uncertain curative effects, low compliance even toxicity. So it is necessary to look for some medicines involving the property of high safety, high efficiency and synthetic therapeutic effects.

Puerarin belongs to a member of flavonoid family, with polyhydroxy (Fig. 1). It is extracted from kudzuvine root and is by far the most important constituent of it, and can be highly effective against angiocardiopathy[13] and cerebrovascular diseases[14] with properties of holding pharmacokinetics of rapid absorption from the intestine and presenting in brain organ tissue by specific transport pathways[15] and low toxicity.[16] Chinese have treated kudzuvine root as food and herb for a long history. Recent researches about kudzuvine root assessed its main active phar-

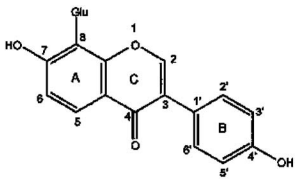


Fig. 1. Structure of Puerarin

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macological component puerarin, especially in health food and cerebrovascular diseases.[17]

The present research shows that puerarin has the actions of dilating the coronary artery[18] and cerebral vessels, increasing cerebral blood flow,[14] modulating vascular endothelial cell function,[19] reducing blood fat and atherosclerosis,[20] scavenging free radical,[21] inhibiting ischemical reperfusion injury[22] and improving hemorheology.[23] Some researches indicate that the neuroprotection of puerarin against cerebral ischemia is associated with the prevention of apoptosis in rats.[14] However, the protecting mechanism of puerarin on the brain neurocytes still remains under study. In addition, the protecting actions of puerarin on the brain neurocytes under the different paths of cerebral ischemia and cerebral ischemia-reperfusion are still not clear. Furthermore, the facts of whether or how puerarin affects some protein expressions involved in cerebral ischemia and cerebral ischemia-reperfusion to protect brain neurocytes in the different conditions of cerebral ischemia still need to be explored. So, this paper was designed to investigate possible roles of puerarin on the expression of HSP70 and Fas protein in rats and explore its protecting mechanism under the level of cells and molecules.

## MATERIALS AND METHODS

**Experimental Animals** Wistar rats (200–300 g, grade of SPF, specific pathogen free) of either sex were employed in the present study. The animals were bought from experimental center of Guangxi Medical University conformed to the standard of China experimental laboratory animal and its license number was SCXK2003–0003. The animal experiments were conducted in accordance with the standard experimental environment of room temperature ($22\pm2$ ℃) and relative humidity (60%–65%). The animals were fed with standard laboratory animal food and qualified water and took in foodstuffs freely.

**Medicines and Reagents** The puerarin injection (2 ml: 100 mg, Guangdong Yantang Biochemistry Pharmaceutical Company, China), HSP70 kit (Fuzhou Maxim Bioengineering Institute, China) and Fas kit (Fuzhou Maxim Bioengineering Institute, China) were prepared. All reagents conform to the national standard within validity duration.

**Instruments** Olympus microscope (Japan), Leica paraffin section machine (Germany), Electrothermal constant temperature incubator (China), Haier refrigerator (China), Microwave oven (China), photomicrograph system (Japan), non-injury artery clamp and microelectrodrill (Germany) were used. All instruments were in good condition.

**The Protection of Puerarin on Acute Cerebral Ischemia and Its Action on HSP70 Expression**

Twelve rats, from the same brood, were matched into 6 pairs in accordance with the same sex and similar weight, then randomly divided into acute ischemia control group and puerarin intervention group. Each group contained 6 cases. Model rats were anaesthetized with chloral hydrate (350 mg/kg$^{-1}$, i.p.). Their skulls were carefully pried open with microelectrodrill and other surgical appliances. Double sides of middle cerebral artery in rats were exposed. One side of middle cerebral artery was in occlusion with non-injury artery clamp for 30 min as ischemic zone, a special region dominated by middle cerebral artery; the other side remained normal flow as non-ischemic zone. Each rat retained control *via* both of its two zones. Rats in puerarin intervention group were injected puerarin (30 mg/kg$^{-1}$, i.p.) for 30 min before arterial occlusion, while rats in acute ischemia control group were injected an equal volume of normal saline with the same methods. Samples from zones of double temporal lobes controlled by middle cerebral arteries in a rat were taken out rapidly in an equal distance and matching quantity and were in formalin fixation for 24 h (4% neutral formalin), routine dehydration, paraffin imbedding and HE staining. Dead neurocyte was judged according to its occurring phenomenon of pyknosis, karyorrhexis and karyolysis. The percentage of dead neurocyte was counted in each slice on visual field under light microscope ($40\times10$) and calculated its mean. The increase value of dead neurocyte was calculated *via* ischemic zone value minus non-ischemic zone value. Staining of HSP70 was conducted with immunohistochemical method. Positive cells were judged by occurring buffy granules in cytoplasm and nucleus. Its intensity was analyzed with pathological image analysis system. Six pictures were randomly collected from each slice ($\times400$) and calculated the mean of grey level. The decreased grey level of Staining of HSP70 was calculated *via* ischemic zone level minus non-ischemic zone level. The more the decreased grey level, the stronger the positive intensity was.

**The Protection of Puerarin on Cerebral Ischemia-reperfusion and Its Action on HSP70 Expression**

Rats, whose experimental methods of animal chosing, grouping, operating, sampling and staining were similar with the previous experiment, were randomly and evenly divided into cerebral ischemia-reperfusion control group and puerarin intervention group. The rat model of cerebral ischemia-reperfusion injury was made by ligatting the middle cerebral artery for 30 min then opened for 30 min. Rats of puerarin intervention group were injected with puerarin in dose of 30 $mg/kg^{-1}$ by intraperitoneal injection before arterial reperfusion 30 min, while rats in control group were injected an equal volume of normal sodium with the same methods. HSP70 expression in brain tissue was detected by SP method of histochemistry as the previous experiment. Dead brain neurocytes were counted and their morphology was observed as the previous experiment.

**The Protection of Puerarin on Cerebral Ischemia and Reperfusion and Its Action on Fas Protein Expression** Thirty matched rats were randomly divided into 5 groups. Each group contained 6 cases. The methods of anesthesia and operation were the same as the above two experiments. Model rats of acute cerebral ischemia and cerebral ischemia-reperfusion were made according to the two methods of previously respective experiments. Methods of administration and dose in four group, ischemia group and ischemia puerarin intervention group, ischemia-reperfusion group and ischemia-reperfusion puerarin intervention group, were the same as the above respective experiments. Normal group was conducted the same operation and injected normal saline, but artery was not occluded. Samples from the zone of operating temporal lobes controlled by middle cerebral artery in rats of various groups were taken out rapidly in an equal distance with equal quantity and were in formalin fixation for 24 h (4% neutral formalin), routine dehydration, paraffin imbedding and HE staining. Expression of Fas protein was conducted with immunohistochemical method. Dead neurocytes were judged according to their occurring phenomenon of pyknosis, karyorrhexis and karyolysis. The percentage of dead neurocytes was counted in each slice on 6 visual fields under light microscope ($40\times10$) and calculated its mean. Positive cells were judged by occurring buffy granules in cytoplasm and cytomembrane. Negative cells were non-stained.

**Immunohistochemical Methods of HSP70 and Fas Protein Expression (SP Method)** Paraffin section is thawed and deparaffinaged, washed 3 times with phosphate buffer saline, 3 min each time, then add CBS liquid and put it into microwave oven to heat for 15 min. Wash it again with phosphate buffer saline as before and add 3% $H_2O_2$ methanol solution to block up endogenous peroxydase for 10 min under room temperature. Wash it again with phosphate buffer saline as before. Add nonimmune animal serum 50 $\mu$l and incubate it for 10 min under temperature of 37℃. Discard serum. Add HSP70 or Fas reagents on each slice and put it into refrigerator (4℃) over night. Wash it again with phosphate buffer saline as before. Add streptavidin-peroxydase solution 50 $\mu$l and incubate it for 10 min at temperature of 37℃. Wash it again with phosphate buffer saline as before. Discard phosphate buffer saline. Add fresh DAB solution 100 $\mu$l and observe it with microscope for 3–10 min. Wash it with water, counterstain with hematoxylin. Mount with neutral gum.

**Statistical Analysis** All the results were expressed as mean ± S.E.M. *T*-test was employed for comparisons between two pairing data groups. One way ANOVA followed by Tukey's test as post hoc test was employed for multiple comparisons between difference groups. $p<0.05$ was considered to be statistically significant.

## RESULTS

**Effects of Puerarin on Acute Cerebral Ischemia and HSP70 Expression** A few dead neurocytes were occasionally found in the non-ischemic zone in both of acute ischemia control groups and puerarin intervention groups, while cerebral edema was not obviously observed in either group (Fig. 2(A)). Dispersed dead neurocytes were observed in the ischemic zone in both of acute ischemia control groups and puerarin intervention groups with obvious cerebral edema. Most dead neurons were pyramidal cells and focal zone of necrosis was not observed. The increased value of dead neurocytes in the puerarin intervention group was significantly less than that in the acute ischemia control group ($p<0.01$) (Table 1). Meanwhile, pathological changes of tissue in the puerarin intervention group were slighter than that in the acute ischemia control group (Figs. 2(B) and 2(C)). These indicated that puerarin can prevent neurocytes from injury in the process of acute cerebral

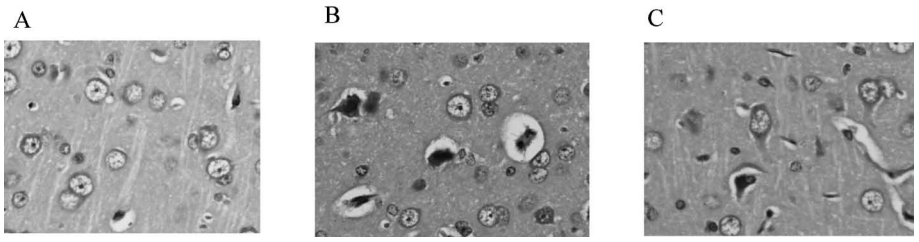


Fig. 2. Illustration of HE Stained Brain Tissue of Rats (HE×400)

A: Feature of histomorphology of non-ischemic zone in acute ischemia control group. B: Feature of histomorphology of ischemic zone in acute ischemia control group, dead neurons were seen. C: Feature of histomorphology of ischemic zone in puerarin intervention group, dead neurons were seen. Cells lesion was slighter than B.

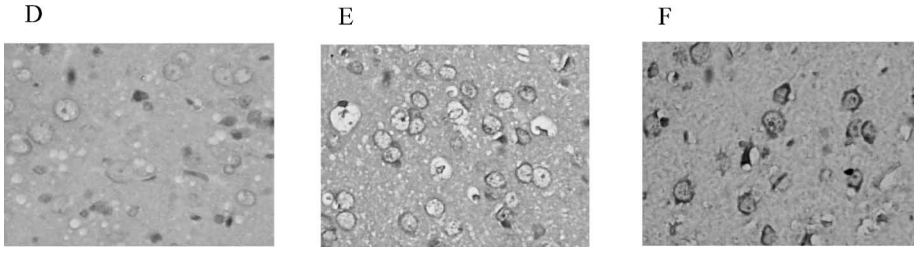


Fig. 3. Illustration of Immunohistochemical Method Stained HSP70 (SP×400)

D: Feature of stained HSP70 of non-ischemic zone in acute ischemia control group. E: Feature of stained HSP70 of ischemic zone in acute ischemia control group, positive in neuron cytoplasm. F: Feature of stained HSP70 of ischemic zone in puerarin intervention group, positive in neuron cytoplasm, stronger than E.

Table 1. Comparison of Dead Neurocytes in Two Groups after Acute Cerebral Ischemia (mean±S.E.M.)

| Group | *n* | increase value of dead neurocytes |
|---|---|---|
| acute ischemia control group | 6 | 13.85±2.37 |
| puerarin intervention group | 6 | 6.78±0.71 |

$p<0.01$.

Table 2. Comparison of Expression of HSP70 in Two Groups after Acute Cerebral Ischemia (mean±S.E.M.)

| Group | *n* | decreased grey level of staining of HSP70 |
|---|---|---|
| acute ischemia control group | 6 | 13.54±3.27 |
| puerarin intervention group | 6 | 29.92±4.58 |

$p<0.01$.

ischemia. Expression of HSP70 presented to be negative or weakly positive in the non-ischemic zone in both of acute ischemia control group and puerarin intervention group (Fig. 3(D)), while the expressions in the ischemic zones in the two groups were all significantly elevated. The expression intensity was stronger in puerarin intervention group than that in acute ischemia control group (Table 2, Figs. 3(E) and 3(F)).

**Effects of Puerarin on Cerebral Ischemia-reperfusion and HSP70 Expression** A few apoptosis neurocytes were occasionally found in the non-ischemia-reperfusion zones in both cerebral ischemia-reperfusion control group and puerarin intervention group, while cerebral edema and hemorrhage were not observed in either group. Dispersed dead neurocytes were obviously observed in the ischemia-reperfusion zones in the two groups with obvious cerebral edema, hyperemia and angioectasia. Pathological changes of tissue were more serious in the cerebral ischemia-reperfusion control group than those in the puerarin intervention group. Furthermore, there were focal zones of necrosis and hemorrhage in cerebral ischemia-reperfusion control group while none of these pathological changes occurred in the puerarin intervention group (Figs. 4(G) and 4(H)). The increased numbers of dead neurocytes in the puerarin intervention group were significantly less than that in the cerebral ischemia-reperfusion control group ($p<0.01$) (Table 3). These indicated that puerarin can prevent neurocytes from injury in the process of cerebral ischemia-reperfusion. Expression of HSP70 presented to be positive in the ischemia-reperfusion zones in both of the groups. In comparison, the ex-

pression of intensity of the puerarin intervention group was much stronger than that of the cerebral ischemia-reperfusion control group ($p<0.01$) (Table 4, Figs. 5(I) and 5(J)).

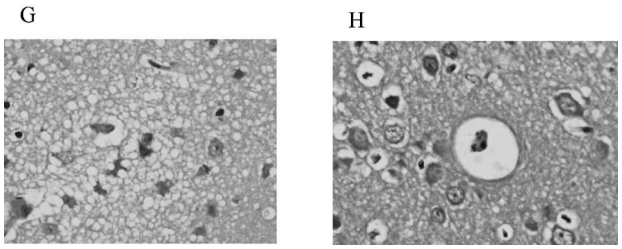


Fig. 4. Illustration of HE Stained Brain Tissue of Rats (HE ×400)

G: Feature of histomorphology of ischemic zone in cerebral ischemia-reperfusion control group. Cellular necrosis and cerebral edema were obviously seen. H: Feature of histomorphology of ischemic zone in cerebral ischemia-reperfusion puerarin intervention group. Cells lesion was slighter than G.

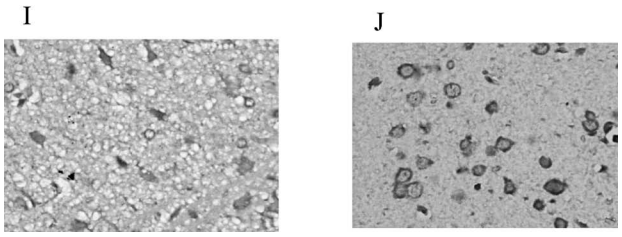


Fig. 5. Illustration of Immunohistochemical Method Stained HSP70 (SP×400)

I: Feature of stained HSP70 of ischemic zone in cerebral ischemia-reperfusion control group, positive in neuron cytoplasm of some cells. J: Feature of stained HSP70 of ischemic zone in cerebral ischemia-reperfusion puerarin intervention group, positive in neuron cytoplasm, stronger than I.

**Effects of Puerarin on Fas Expressions in Acute Cerebral Ischemia and Cerebral Ischemia-reperfusion**

Fas protein can be expressed in some degree in all groups. Most of the positive cells were neurons and the strongest expressive intensity lays in pyramidal cells (Figs. 6(K), 6(L), and 6(M)). Compared with the normal group, the ischemia group had a trend of expressing Fas stronger while the ischemia puerarin intervention group had a trend of expressing Fas weaker, but there were no significant differences among the three groups ($p>0.05$). Both the ischemia-reperfusion group and the ischemia-reperfusion

Table 3. Comparison of Dead Neurocytes in Two Groups after Cerebral Ischemia-reperfusion (mean±S.E.M.)

| Group | *n* | increased value of dead neurocytes |
|---|---|---|
| cerebral ischemia-reperfusion control group | 6 | 42.76±17.76 |
| puerarin intervention group | 6 | 10.88±1.58 |

$p<0.01$.

Table 4. Comparison of Expression of HSP70 in Two Groups after Cerebral Ischemia-reperfusion (mean±S.E.M.)

| Group | *n* | decreased grey level of staining of HSP70 |
|---|---|---|
| cerebral ischemia-reperfusion control group | 6 | 17.97±6.49 |
| puerarin intervention group | 6 | 35.08±2.47 |

$p<0.01$.

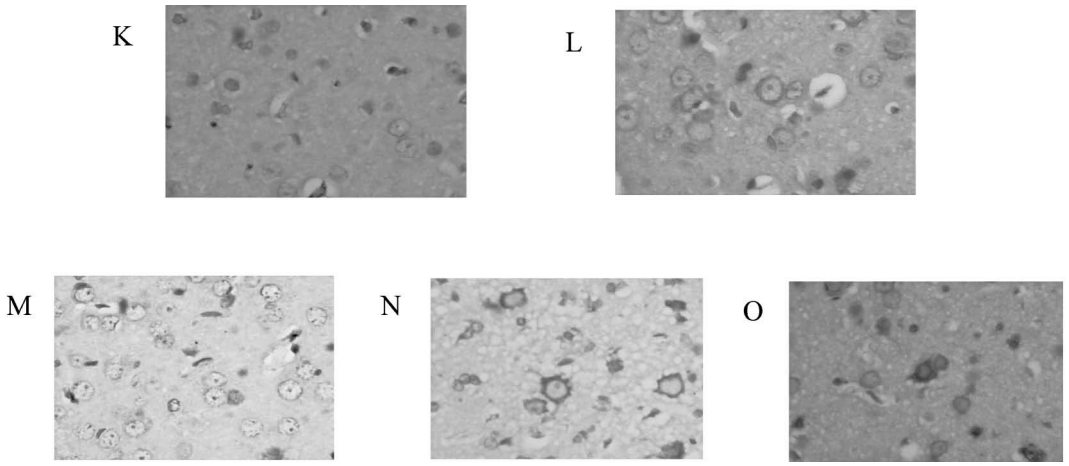


Fig. 6. Illustration of Immunohistochemical Method Stained Fas Protein Expression (SP×400)

K: Feature of stained Fas protein expression in normal group, tenuously positive. L: Feature of stained Fas protein expression in ischemia puerarin intervention group, tenuously positive. M: Feature of stained Fas protein expression in ischemia group, tenuously positive. N: Feature of stained Fas protein expression in ischemia-reperfusion group, strongest positive. O: Feature of stained Fas protein expression in ischemia-reperfusion puerarin intervention group, positive.

Table 5. Comparison of Expression of Fas among Five Groups after Acute Cerebral Ischemia and Cerebral Ischemia-reperfusion (mean±S.E.M.)

| Group | *n* | rate of Fas positive cells |
|---|---|---|
| normal group | 6 | 5.85±1.25[1,2)] |
| ischemia group | 6 | 6.16±1.31[1,2)] |
| ischemia puerarin intervention group | 6 | 5.88±0.99[1,2)] |
| ischemia-reperfusion group | 6 | 75.33±4.16 |
| ischemia-reperfusion puerarin intervention group | 6 | 34.68±2.56[1)] |

*VS* ischemia-reperfusion group, [1)] $p<0.01$.
*VS* ischemia-reperfusion puerarin intervention group, [2)] $p<0.01$.

puerarin intervention group can express Fas stronger than each of the other ones can ($p<0.01$). In comparison, the express in the ischemia-reperfusion puerarin intervention group was weaker than that in the ischemia-reperfusion group ($p<0.01$) (Table 5, Figs. 6(N) and 6(O)).

## DISCUSSION

**Injury Mechanism of Neurocyte in Acute Cerebral Ischemia and Cerebral Ischemia-reperfusion and the Protection of Puerarin** The physiopathologic course of neurocyte injuries after cerebral ischemia and ischemia-reperfusion is very complicated. Cerebral ischemia causes hypoxia of neurocytes which are sensitive to hypoxia and inhibits oxidative phosphorylation of chondrosome in neurocytes. As a consequence, production of ATP decrease and cell energy burns out. Meanwhile, depolarization of cell membrane causes the release of glutamic acid increased, excitatory amino acid (EAA) receptor is activated. It triggers a huge inflow and release of $Ca^{2+}$ and cause intracellular calcium overload, changes various enzymatic activity, especially activating proteinase, such as calcineurin and calpain.[24)] When activated, they can change chromosomal structures, activate nucleate endonuclease, trigger DNA fragmentation, lead to neuron apoptosis after ischemia. On the other hand, due to the low function of the calcium pump, calcium overload deteriorates and encourages the production of a great deal of free radicles, which aggravate neuron apoptosis. Though blood reperfusion in the early state of ischemia may retrieve part of ischemic tissue, it continues to cause neuron injuries, even deteriorates the injuries and leads to death or apoptosis. These adverse consequences caused by ischemia-reperfusion have been termed as reperfusion injury or reperfusion trauma.[25)] Recent study showed that the expression of gene bcl-2 tapered off with extending the duration of cerebral ischemia-reperfusion and increasing cells of apoptosis. This indicates the protection of gene bcl-2 in cerebral ischemia-reperfusion.[26)]

It is reported that puerarin exerted the protective action on injured neurocytes due to its mechanism of inhibiting $Ca^{2+}$ aggregation, lessening cell injuries, up-regulating the expression of gene bcl-2 and down-regulating the expression of Bax protein and inhibiting neurocyte apoptosis.[27)] Our results showed that puerarin can prevent neurocytes from injury in both rat model of acute cerebral ischemia and cerebral ischemia-reperfusion. It can significantly reduce the number of dead neurocytes and lessen the degree of cerebral edema and hemorrhage. These offered morphological evidence to protective mechanism of puerarin on ischemic injured neurocytes.

Traditionally, dead neurocytes act as the property of necrosis.[28)] Recently, some studies, from the view of morphology, biochemistry, materia medica and gene, proved that apoptosis existed in the process of cerebral ischemia-reperfusion.[29,30)] Further studies showed that the emergence time and numbers of apoptosis related to experimental animal brood, location and ischemic degree.[31–33)] Apoptosis is the initiative cell death regulated by multiple genes and triggered by reserved dead procedure. Cell necrosis is a passive death induced by some pathological stimulating factor such as oxygen deficiency and toxicosis. Dead scopes of cells in apoptosis display sporadic single cell while these in necrosis mainly show congregate cell mass. However, the two cell death ways still overlap in some death mechanisms and morphology. Free radicals in low concentration can induce apoptosis while causing necrosis in high concentration. Besides in necrosis, karyopycnosis, karyorrhexis and chromatinic gather to the edge and also are found in apoptosis.

On our study in this paper, we aimed at the comparison with neurocytes injuries between groups, so we selected the sign of dead neurocytes as the comprehensive assessment. As for how to differ from apoptosis and necrosis on the protection of puerarin in acute cerebral ischemia and cerebral ischemia-reperfusion, we will further conduct a series of studies to explore them on the level of cell and gene.

**HSP70 Expression after Acute Cerebral Ischemia and Cerebral Ischemia-reperfusion and the Effect of Puerarin** Since 1996, Sato[34] first observed the expression of heat shock protein on the cellular level, it has been studied on the structure and function. HSP70, a member of the family of heat shock protein, is the most conservative protein in the process of organic evolution. HSP70 is exhibited strongly under the condition of stress stimulus and is considered to be plasticity gene. HSP70 is known to have the function of molecular chaperones. It helps degenerated proteins restore and helps newly synthetic polypeptide bond and physiologically fold and unfold. HSP70 has a high affinity to ATP and make use of its energy to undo error fold of polypeptide bond in degenerated protein, therefore make it possible to be a normal protein, which can recover the structure and function of the injured cell. HSP70 is still necessary to retroposition after ribosome assembly and improves the synthesis of newly born ribosomes and proteins. It accelerates the recovery of cell functions. In addition, HSP70 can combine to cytoskeletal protein, protect its structure and prevent it from degeneration. So, maintaining a certain level of HSP70 in the neurocytes can be considered to be a new method of brain protection in rats with cerebral ischemia.[35] Recent researches found that HSP70 is the important endogenous substance of anti-injury in cerebral ischemia and inducing HSP70 high expression may be one of the endogenous protective mechanism in the brain.[36,37] Its main function was to prevent the ischemic cell from injury caused by various stress. Medicine inducing HSP70 high expression can lessen neuron injuries in ischemia and ischemia-reperfusion. This indicates that medications such as DF-521 (Batroxobin) and Ligustrazine enhancing HSP70 production show value and perspectives on prevention and treatment in neurocytes of ischemia and ischemia-reperfusion.[38,39]

Current studies reported that puerarin protected the neurocyte from injury mainly through checking aggregation of calcium ions and lessening cell injury. These had a correlation with its actions of enhancing bcl-2 protein expression and inhibiting Bax protein expression and reducing apoptosis.[27,36]

Our studies show that puerarin can lessen injured neurocytes and reduce their rates of dead cells in both of the durations of ischemia and ischemia-reperfusion. Morphological studies in cells further show the same protective effects. Meanwhile, puerarin can induce high expression of HSP70 significantly in both of the different ischemia periods. Therefore it may be concluded that puerarin can protect the brain neurocyte of rat in acute local ischemia brain injury and local cerebral ischemia-reperfusion injury through one of the mechanism of enhancing HSP70 production, which improves the endurance of stress and therefore reducing apoptosis.

**Fas Protein Expression after Acute Cerebral Ischemia and Cerebral Ischemia-reperfusion and the Effect of Puerarin** Neurocytes injuries after acute ischemia and ischemia-reperfusion relate to brain tissue releasing oxygen free radical and NO (nitrogen monoxidum), which lead to cell necrosis and apoptosis. Protien kinase C$\delta$ can hasten apoptosis of various cells, including neurocytes.[40–43] Apoptosis, a main cause of injuries in ischemia-reperfusion, is mediated by multi paths. System of Fas and Fas ligand is known as one of the main paths. Fas is a molecule of cell surface protein, containing a segment of death domain composed of 60–70 amino acid sequences. When combined with death-associated protein, Fas activates Caspase-8, triggers a series of Caspase enzyme reaction, causing DNA degradation and apoptosis. Fas acts as an important controlling gene to enhance apoptosis. It belongs to a member of family of tumor necrosis factor/nerve growth factor. Its expression products transmit a signal of apoptosis.[44,45]

A study concerning global brain ischemia in rat model showed that puerarin reduced apoptosis in brain resuscitation due to its inhibiting Fas expression, but apoptosis significantly decreased after elapsed time of 3 h after ischemia-reperfusion. This indicated that puerarin reduced apoptosis in brain resuscitation involving other complicated regulation of genes.

Our results showed that there were no significant differences between groups of ischemia and normal group, while puerarin reduced the dead rate of neurocytes and enhanced HSP70 expression. These hinted that the protection of puerarin in ischemia related to the action of HSP70 expression while not linked to Fas expression. In the period of ischemia-reperfusion, our results showed that Fas expressed strongly in both of ischemia-reperfusion group and ischemia-reperfusion puerarin intervention group compared with ischemia groups and normal group. These manifested that Fas expression was involved in pathological

change in the duration of ischemia-reperfusion. We still observed that puerarin significantly reduced Fas expression in the period of ischemia-reperfusion. Basing on these facts, we may draw a conclusion that puerarin exerts protective effects on neurocytes through enhancing HSP70 expression in the period of ischemia while taking effect *via* not only enhancement HSP70 expression but inhibiting Fas expression over the duration of ischemia-reperfusion.

This paper shows that puerarin holds the different protecting properties on neurocytes during the different periods of cerebral ischemia. During acute cerebral ischemia, neurocytes injure due to dysmetabolism, which leads to cell degeneration and necrosis, While, apoptosis mediated by Fas protein does not contribute a lot to the injuries. So, puerarin protects the neurocytes mainly by enhancing HSP70 expression and therefore improving the level of stress of neurocytes in this period. During cerebral ischemia-reperfusion, neurocytes are injured due to double mechanisms of dysmetabolism and apoptosis. Puerarin therefore, protects the neurocytes through enhancing HSP70 expression and Fas protein expression during this time. As a result, puerarin acts both to improve the level of stress of neurocytes and reduce neurocyte apoptosis to protect neurocytes.

The death of neurons, involved in multiple mechanisms, is described as two different paths: apoptosis and necrosis, which respectively refers to active and passive mechanisms.[46] The dead neurons and glial cells are quickly accumulating in the center area of ischemia after a couple of minutes of postischemia.[47] Meanwhile, around this area gradual apoptosis occurred, and the numbers of them increased with prolonged time.[48] Li's researches found that 10–20 apoptosis neurons appeared on one coronal section of 5 $\mu$m thickness after 10–20 minutes' ischemia-reperfusion in rats by ligating middle cerebral artery, distributing on the selective zones of necrosis, preoptic region and corpus striatum. similarly, 30–60 apoptosis neurons distributed on cortical selective zones of necrosis after 30–60 minutes' ischemia-reperfusion and 70–200 ones on posterior margin of cortex, corpus striatum, hippocampus and olfactory tubercle after 90–120 minutes' ischemia-reperfusion.[49,50] This indicates that apoptosis after cerebral ischemia and ischemia-reperfusion is a developing dynamic process. In early ischemia, it mainly manifests neuronal necrosis with a few apoptosis cells while it occurs a great quantity of apoptosis cells during the later period of ischemia and ischemia-reperfusion.

In the conditions of our experiment, the period of acute ischemia and ischemia-reperfusion respectively lasts only 30 min. Compared with the literature, delayed neuronal death (DND) reaching its peak after ischemia of 1–5 days and lasts about 4 weeks,[48] the time course of our experiments is shorter. Thus, neuronal necrosis was mainly observed during ischemia period and necrosis and apoptosis were concomitant during ischemia-reperfusion period in the present paper. This rarely involved delayed neuronal death caused by releases of cytokines from focal necrosis, such as TNF-$\alpha$, IL-1$\beta$, IL-6 *etc.*

These facts further explain and confirm our results. In the process of acute ischemia, the protecting effect of puerarin mainly relates to up-regulation of HSP70 production while in the period of ischemia-reperfusion, it relates to both processes of up-regulation of HSP70 expression and reduction of Fas expression.

Our results show that the number of cells expressed Fas protein positively in each group has no correlation with that of dead cells. This indicates that the path of cell death is mediated not only by Fas, but by others. It further proves the complexity of neurocytes injuries in cerebral ischemia.

## CONCLUSIONS

Puerarin has the protective actions in both processes of acute cerebral ischemia and cerebral ischemia-reperfusion. The protecting mechanism varies with the difference of neurocyte injuries.

In the process of acute cerebral ischemia, the protecting effect of puerarin mainly relates to up-regulation of HSP70 expression, while not correlated to the expression of Fas protein.

In the period of cerebral ischemia-reperfusion, the protecting effect of puerarin relates to both processes of up-regulation of HSP70 expression and down regulation of Fas expression.

The neuroprotective effects of puerarin in the duration of acute cerebral ischemia exerts the actions of improving the level of stress and promoting the recovery of degenerated protein. During cerebral ischemia-reperfusion, puerarin acts on not only these effects, but reducing neurocyte apoptosis.

**Acknowledgements** We would like to thank the staff of the Science Fund Committee of Guangxi Zhu-

ang Autonomous Region of China for their help in making this research successful. We especially thank the leader of People's Hospital of Guangxi Autonomous Region of China for his various supports in conducting this research. We especially thank U.S.A. advanced English experts, Ms. Lois and Mr. Kiefer for their rigorous and serious revisions in making this article more readable and understandable. We also thank the reviewers of this journal for their comments in making this article more precise.

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