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Protective effects of quercetin against status epilepticus induced hippocampal neuronal injury in rats: involvement of X-linked inhibitor of apoptosis protein

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Abstract

Epilepsy is a common neurological disorder affecting people worldwide, and the recurrent spontaneous seizures are often seen post status epilepticus. Apoptosis and necrosis are two forms of neuronal death in post status epilepticus hippocampus, and the former has been widely studied and believed to be a major factor contributing to formation of abnormal excitatory circuit leading to refractory epileptic events. Thus, the need for development of new anti-epileptic agents remains urgent. Quercetin, a plant-derived bioflavonoid, is reported to have neuroprotective effects in neurological disease. We investigated protective effects of quercetin on status epilepticus induced hippocampal neuronal injuries in rats and focused on two major proteins, the X-linked inhibitor of apoptosis protein, a key member of the inhibitor of apoptosis protein family, and the caspase-3 protein, a common effector for the execution-phase of cell signaling apoptotic pathways. The number of apoptotic and surviving neurons were also counted in this study. We found expression alterations of X-linked inhibitor of apoptosis protein and caspase-3 protein in post status epilepticus hippocampus, along with an alteration in the number of apoptotic and surviving neurons. Furthermore, quercetin treatment in rats undergoing status epilepticus led to an interventional effect on expression of X-linked inhibitor of apoptosis protein and the caspase-3 protein, with a corresponding positive change on the number of hippocampal apoptotic and surviving neurons. Together, the study suggests neuroprotective effects of quercetin on hippocampal injury post status epilepticus and the effects may be associated with regulation of the X-linked inhibitor of apoptosis protein and the caspase-3 protein, which can be a decisive factor for apoptosis and survival of neurons in hippocampus.

Key words: Status epilepticus; quercetin; X-linked inhibitor of apoptosis protein; apoptosis; hippocampus.

Introduction

Epilepsy is the most common neurological disorder that affects people of all ages and there are about 50 million people suffering from epilepsy worldwide. Although previous studies have achieved much progress in the basic research of epilepsy, its molecular basis and the pursuit for effective anti-epileptic drugs still need research efforts. Interestingly, in addition to many chemical substances whose anti-epileptic properties have already been validated, plant may serve as an alternative resource for development of new anti-epileptic agents.

Quercetin, a flavonoid compound with anti-oxidative abilities, owns protective effects against ischemia-induced brain injury and can be used to improve cognitive impairment. Moreover, a previous research suggested that quercetin prevents tissue damage through various mechanisms, for example by inhibiting neuronal apoptosis in the brain (Bureau *et al*., 2008). Although hippocampal neuronal death post status epilepticus (SE) has been widely accepted, it is not clear whether quercetin treatment could reduce SE-induced neuronal injury in the hippocampus and which molecular mechanisms could mediate this effect.

Recent research evidence has demonstrated that the process of apoptosis can be regulated at several checkpoints in apoptotic signaling pathway. Among those cellular proteins that have been proved to regulate caspase cascades activation is the family of Inhibitor of apoptosis protein (IAPs), in which X-linked inhibitor of apoptosis protein (XIAP) is recognized as the most potent caspase inhibitor (Liston *et al*., 1996). XIAP directly bind and inhibit specific members of the caspase family including caspase-3, -7 and -9 (Holcik *et al*., 2001), negatively

regulating caspase cascades and inhibiting the apoptotic process. Previous experimental evidence has confirmed the role of XIAP regulation in rat hippocampus following transient forebrain ischemia (Siegelin MD *et al*., 2005), indicating the involvement of XIAP in neurological disorders. However, reports concerning possible association of XIAP in epilepsy are limited.

As neuronal apoptosis takes the primary form of neuronal death in hippocampus post-SE, we hypothesize that XIAP, as an important molecular regulator for apoptosis, may be involved with hippocampal neuronal injury led by SE and act as an apoptotic regulator as it does in other neurological disease. Moreover, in addition to its intrinsic antioxidative abilities, we hypothesize that quercetin may alleviate SE-induced injury in hippocampus partly due to an effect on XIAP, and that will have ultimately kept the hippocampal neurons away from advancing more deeply into cellular apoptotic stage. To elucidate this question, we established classical rat lithium-pilocarpine SE model and assessed neuro-protective effects of quercetin by evaluating the XIAP expression, the activated caspase-3 protein expression and an account of apoptotic and surviving neurons in post-SE rat hippocampus.

Materials and methods

Animals and grouping

Male Sprague-Dawley rats(weighing 230 g-270 g, from animal unit at Central South University, China) were randomly assigned to three groups: the control group, the SE group and the quercetin-treated SE group. The SE group or the quercetin-treated SE group were each randomized into five sub-groups (2 h, 4 h, 8 h, 24 h and 72 h post-SE). For each time point in each sub-group and control group, there were n = 6 rats used for Western blot and RT-PCR analysis, and another n = 6 rats for immunohistochemistry, TUNEL and Nissl staining. The rats used: Control group (6 × 2), SE group (6 × 5 × 2), Que-treated SE group (6 × 5 × 2), there were a total of 132 rats. Experiments were carried out in accordance with NIH Guide for Care and Use of Laboratory Animals.

SE model and quercetin treatment

The SE model was established by intraperitoneal injection of lithium chloride and pilocarpine. Lithium chloride (125 mg/kg, i.p., Sigma) was injected 18 h-20 h prior to administration of pilocarpine (10 mg/kg, i.p., Sigma). SE was defined as the onset of continuous generalized (stage 4-5 by Racine's scale) seizure activities lasting no less than 60 min and not having regained normal behavior between seizures (Francois J *et al*., 2006). The rats in the quercetin-treated SE group received quercetin treatment (50 mg/kg, i.p. purchased from National Institutes for Food and Drug Control of China) 15 min after SE onset. All SE rats received chloral hydrate (10%, 3 ml/kg, i.p.) to terminate epileptic attacks.

Immunohistochemistry

Brain tissues were formalin-fixed and paraffin-embedded. Immunohistochemical staining was performed by SABC method (Boster Co., China). The primary antibody included a goat anti-rat XIAP polyclonal antibody (1:200, R&D Co., US) and a mouse anti-rat caspase-3 monoclonal antibody (1:200, Abcam). Sections were later incubated with biotinylated secondary antibodies (1:200, Boster Co., China) before processed with the HPIAS-1000 Color Image Analysis System to measure optical density (OD) values of those positive immuno-reactive products (protein/antibody complexes). For each section, 5 non-overlapping visual fields in bilateral hippocampal CA3 regions were selected per hyperfield (× 400) and analyzed for OD values, and for each rat 3 hippocampal sections were examined before attaining mean OD values for statistics.

Western blot analysis

The cytoplasmic extracts were separated by 12% SPS-PAGE and transferred onto a PVDF membrane using the Bio-Rad system (Bio-Rad, USA), and then blocked with TBST containing 5% nonfat milk for 2 hours at room temperature and gentle shaking. Three washing steps of 10 min each were carried out after blocking and incubations with antibodies. All primary antibodies were diluted in blocking buffer at appropriate concentrations of 1:1000 according to antibody instructions. The primary antibodies include a Goat anti-Rat XIAP polyclonal antibody (R&D Systems, USA) and a Mouse anti-Rat Caspase-3 monoclonal antibody (ABCAM, USA). The GAPDH expression was used as internal control. The membrane was incubated overnight at 4°C with the primary antibodies, followed by incubation with horseradish-peroxidase-conjugated anti-Goat and anti-Mouse secondary antibodies (Zymed, USA) using 1:2000 dilution at room temperature for 1 hour. The protein/antibody complexes were detected using chemiluminescence reagents (ECL) (KPL, USA). Optical density (OD) values were measured.

The relative expression amounts of XIAP and the caspase-3 protein were evaluated by ratios of XIAP/GAPDH and caspase-3 protein/GAPDH.

RT-PCR

The Total RNA was extracted by using the Trizol Kit (Molecular Research Center, U.S.A). cDNA was then synthesized from 1 μg total RNA by Reverse Transcription Kit (TOYOBO Co., Japan). PCR was carried out in a best-optimized reaction volume of 25 μl and β-actin was used as the internal control. Optical density (OD) values of the RT-PCR products were measured and the relative expression amount of XIAP mRNA was evaluated by the ratio of XIAP/β-actin. Primers were synthesized by Invitrogen Co., China. XIAP-Forward: 5'-AGGAACCCT-GCCATGTATTG-3', XIAP-Reverse: 5'-TGTTGTT CCCAAGGGTCTTC-3'. β-actin-Forward: 5'-CG-CACCACTGGCATT GTCAT-3', β-actin-Reverse: 5'-TTCTCCTTGATGTCACGCAC-3'.

TUNEL assay and Nissl staining

TUNEL assay was performed to detect apoptotic neurons. The procedures were carried out according to the manufacturer's instructions (Roche Co., Germany). The TUNEL-positive cells were apoptotic neurons, defined as those ones displaying buffy particles in their nucleus. Nissl staining was performed to detect surviving neurons. The sections were stained with 0.5% Cresyl violet (w/v) for 10 min before they were dehydrated and mounted with permount. The CA3 hippocampal region from each rat was captured by microscope (Olympus, Japan). The apoptotic neurons and surviving neurons were evaluated by the neurons counted per hyperfield. For each section, 5 non-overlapping visual fields in bilateral hippocampal CA3 regions were selected per hyperfield (× 400) in order to calculate the number of apoptotic or surviving neurons, and for each rat 3 hippocampal sections were examined before mean numbers were attained for statistics.

Statistics

Statistical analysis was performed using the SPSS 12.0 software. All data were expressed as mean ± standard deviation. Differences between multiple groups were statistically assessed by one-way ANOVA analysis. Differences between two groups at the same time point were evaluated by independent-samples t-test. For all statistical analyses, $p < 0.05$ was considered statistically significant.

Results

The alteration of XIAP mRNA and XIAP expression

The expression of XIAP mRNA and XIAP was significantly increased in post-SE rat hippocampal CA3 region. As shown in Table 1 and Fig. 1A, the RT-PCR detection for XIAP mRNA in rats undergone SE had demonstrated a pattern of increase at the time points of 2 h, 4 h, 8 h and 24 h post-SE when compared with rats in the control group. Moreover, a similar pattern of expressional increase at the same time points had been observed when using immunohistochemistry to detect quantitiy and space distribution of XIAP post-SE (Table 1 and Fig. 2A, Fig. 2B), although Western blot results (Fig. 3A) just showed an increasing trend for the XIAP expression but had not confirmed the statistical significance for differential expression between control group and groups of different time points. The reason for a lack of differential expression of XIAP by western blot analysis in our results as described above has been elucidated clearly in the discussion section.

Moreover, the expression of XIAP mRNA and XIAP in the quercetin-treated SE group demonstrated a significant expressional increase at the time points of 8 h, 24 h in post-SE rat hippocampal CA3 region, when compared with the expression of XIAP mRNA (Table 1 and Fig. 1B) and XIAP (Table 1 and Fig. 2B, Fig. 2C) in the SE group. The western blot results (Fig. 3C) had confirmed this alteration of XIAP expression at time points of 8 h and 24 h just as that had been detected by immunohistochemistry.

The alteration of the caspase-3 protein expression

The expression of the caspase-3 protein was significantly upregulated in post-SE rat hippocampal CA3 region. As shown in Table 1, no caspase-3 protein expression had been detected in post-SE rat hippocampal CA3 region in the control group, but it had showed a markedly expression increase at different time points in the same region post-SE (Table 1 and Fig. 2D, Fig. 2E). Western blot results had further confirmed this alteration of the caspase-3 protein expression in post-SE hippocampus (Fig. 3B).

Furthermore, the expression of the caspase-3 protein in the quercetin-treated SE group demonstrated a significant expression decrease at the time points of 8 h, 24 h in post-SE rat hippocampal CA3 region, when compared with expression of caspase-3 protein (Table 1 and Fig. 2E, Fig. 2F) in the SE group. The western blot results (Fig. 3D) had also confirmed this alteration of the caspase-3 protein expression at time

Table 1

XIAP mRNA, XIAP, Caspase-3 protein expression, and the number of TUNEL-positive neurons and surviving neurons in post-SE rat CA3 hippocampal region (± s, n = 6)

Group	*XIAP mRNA*	*XIAP*	*Caspase-3*	*TUNEL-positive Neurons*	*Surviving Neurons*
Control Group	0.2564 ± 0.0251	0.1507 ± 0.0165	–	–	113.12 ± 17.02
SE Group 2 h	0.5542 ± 0.0186[a]	0.5503 ± 0.0172[a]	0.2103 ± 0.0113	9.00 ± 6.21	102.36 ± 14.10
4 h	0.5773 ± 0.0327[a]	0.6029 ± 0.0493[a]	0.3463 ± 0.0191	17.62 ± 3.87	73.63 ± 16.11[a]
8 h	0.6328 ± 0.0329[a]	0.6221 ± 0.0238[a]	0.6967 ± 0.0110	61.80 ± 5.13	60.74 ± 16.24[a]
24 h	0.4195 ± 0.0341[a]	0.5095 ± 0.0374[a]	0.9542 ± 0.0162	86.45 ± 5.21	55.08 ± 14.36[a]
72 h	0.3897 ± 0.0165	0.3076 ± 0.0197	0.7925 ± 0.0219	68.25 ± 4.15	49.16 ± 13.87[a]
Que Group 2 h	0.5560 ± 0.0229	0.5567 ± 0.0318	0.2071 ± 0.0196	6.38 ± 3.76	108.38 ± 16.14
4 h	0.5794 ± 0.0197	0.6034 ± 0.0322	0.3068 ± 0.0216	15.74 ± 4.42	90.64 ± 14.11
8 h	0.8043 ± 0.0342[b]	0.8042 ± 0.0576[b]	0.5432 ± 0.0170[b]	46.33 ± 3.15[b]	84.67 ± 13.65[b]
24 h	0.5610 ± 0.0268[b]	0.7183 ± 0.0168[b]	0.7995 ± 0.0195[b]	55.76 ± 6.18[b]	78.26 ± 16.06[b]
72 h	0.3738 ± 0.0147	0.3276 ± 0.0192	0.6454 ± 0.0241[b]	49.28 ± 5.17[b]	51.47 ± 15.27

Notes: All values are expressed as mean ± SEM.

[a] $P < 0.01$ vs control group.

[b] $P < 0.01$ vs SE group, quercetin 50 mg/kg treated. Que: quercetin.

i) XIAP mRNA expression was evaluated by the ratio of XIAP mRNA/β-actin mRNA.

ii) XIAP and Caspase-3 protein expression were evaluated by their optical density (OD) values / per hyperfield (× 400) , respectively.

iii) The number of TUNEL-positive (apoptotic) neurons or surviving neurons in post-SE rat CA3 hippocampal region was evaluated by the neurons counted / per hyperfield (× 400).

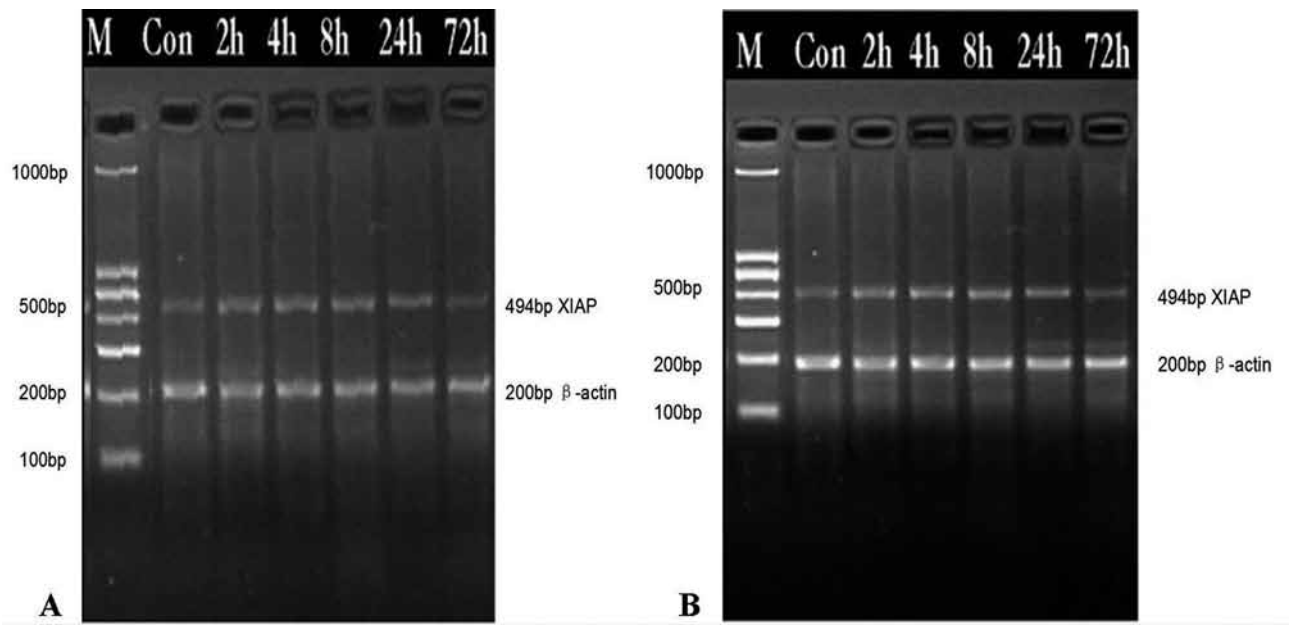

FIG. 1. — Electrophoresis of XIAP mRNA.

Fig. 1A-B show the expression of XIAP mRNA in the SE group and the quercetin-treated SE group, respectively. The expression of β-actin mRNA is also shown in Fig. 1A-B.

M: marker; Con: control group.

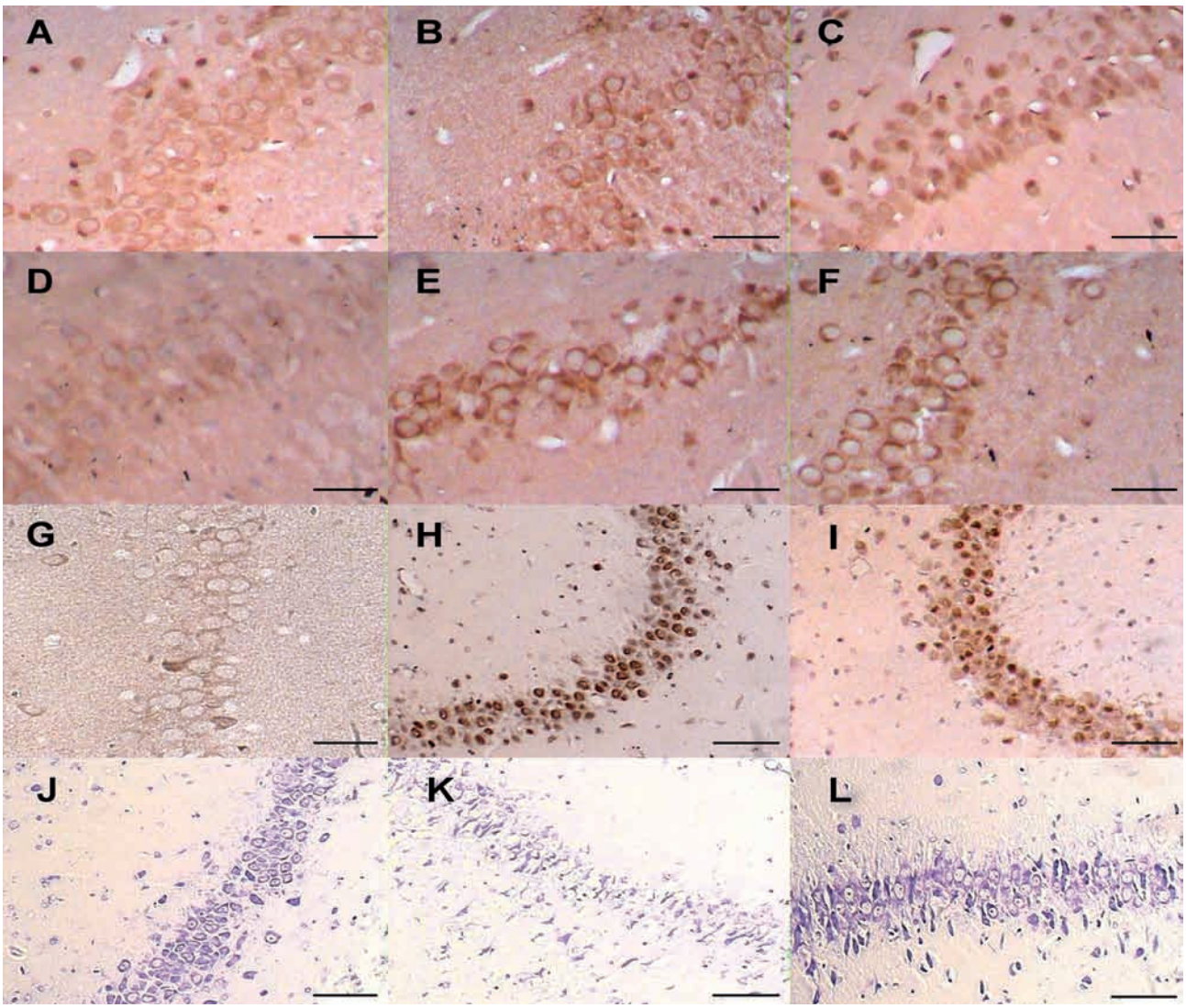

FIG. 2. — XIAP and Caspase-3 protein detection, TUNEL-positive neurons and surviving neurons detection in rat hippocampal CA3 region. (Time point = 8 h post-SE; Scale bars = 100 μm)

Notes: Fig. 2A\2D\2G\2J: control; Fig. 2B\2E\2H\2K: SE 8 h; Fig. 2C\2F\2I\2L: Quercetin 8 h.

i) XIAP detected was shown in Fig. 2A-C. The XIAP expression significantly increased in the Que 8h group (Fig. 2C) when compared with that in the SE 8 h group (Fig. 2B).

ii) Caspase-3 protein detected was shown in Fig. 2D-F. The caspase-3 protein expression was significantly inhibited in the Que 8 h group (Fig. 2F) when compared with that in the SE 8 h group (Fig. 2E).

iii) TUNEL-positive neurons were shown in Fig. 2G-I. The number of TUNEL-positive neurons decreased in the Que 8 h group (Fig. 2I) when compared with that in the SE 8 h group (Fig. 2H).

iv) Surviving neurons were shown in Fig. 2J-L. The number of surviving neurons increased in the Que 8 h group (Fig. 2L) when compared with that in the SE 8 h group (Fig. 2K).

points of 8 h and 24 h just as that had been detected by immunohistochemistry.

ANALYSIS ON THE NUMBER OF APOPTOTIC NEURONS AND SURVIVING NEURONS

The number of apoptotic neurons was markedly increased whereas the number of surviving neurons was significantly decreased in post-SE hippocampus. As shown in Table 1, there were no apoptotic neurons detected in hippocampal CA3 region in the control rats, but the number of apoptotic neurons increased significantly at different time points in the same region post-SE (Table 1 and Fig. 2G, Fig. 2H). Meanwhile, the number of surviving neurons decreased markedly at the corresponding time points in post-SE rat hippocampal CA3 region (Table 1 and Fig. 2J, Fig. 2K).

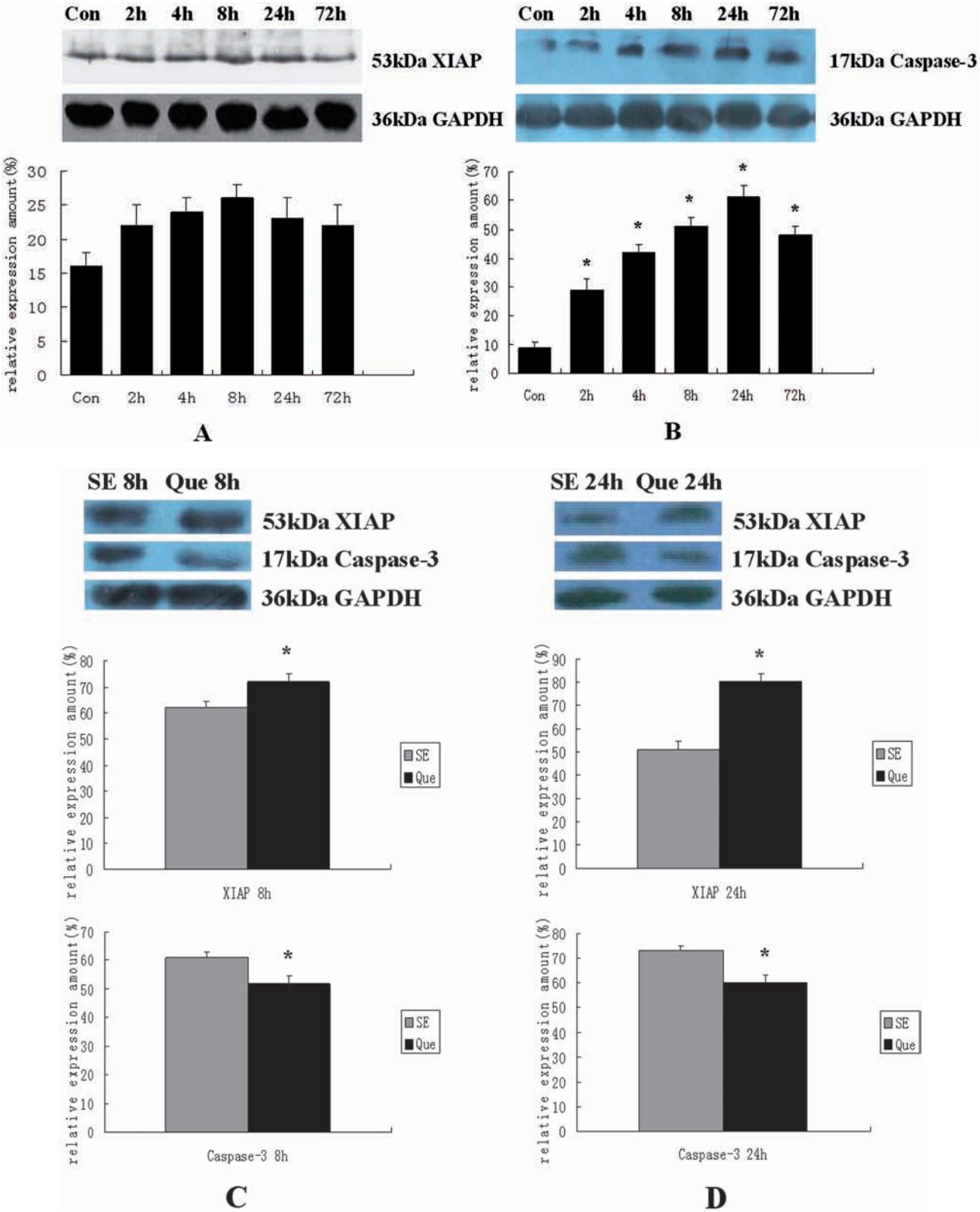

FIG. 3. — Western blot analysis of XIAP and caspase-3 protein expression.
Fig. 3A: XIAP expression at different time points post-SE. $P > 0.05$, vs. control; n = 6.
Fig. 3B: Caspase-3 expression at different time points post-SE. *$P < 0.01$, vs. control; n = 6.
Fig. 3C: XIAP and Caspase-3 expression at the time point of 8 h after quercetin treatment. *$P < 0.01$, vs. SE 8 h; n = 6.
Fig. 3D: XIAP and Caspase-3 expression at the time point of 24 h after quercetin treatment. *$P < 0.01$, vs. SE 24 h; n = 6.

Moreover, the number of apoptotic neurons was significantly decreased whereas the number of surviving neurons was markedly increased after quercetin treatment in post-SE hippocampus. As can be seen in Table 1 and Fig. 2H, Fig. 2I, the number of apoptotic neurons decreased significantly at the time points of 8 h, 24 h and 72 h after quercetin treatment in post-SE rat hippocampal CA3 region when compared with that in the SE group. Meanwhile, the number of surviving neurons increased markedly at the corresponding time points of 8 h and 24 h after quercetin treatment in post-SE rat hippocampus (Table 1 and Fig. 2K, Fig. 2L) when compared with that in the SE group.

Discussion

Apoptosis is widely recognized as one characteristic of SE-induced hippocampal neuronal injuries (Kondratyev *et al*., 2004), whereas the other feature of hippocampal neuronal injuries led by SE is necrosis. Despite a number of studies depicting these two traits in hippocampus post-SE, however, there is presently still a lack of strigent standard that distinguishes between neuronal apoptosis and necrosis. Under specific circumstances, neuronal death in certain brain regions takes the main form of necrosis (Fujikawa DG *et al*., 2000), while in other conditions, evidence for neuronal apoptosis is strong and clear (Kondratyev *et al*., 2004). No matter what kind of forms neuronal death has adopted, processes of energy deprivation, mitochondrial failure, degradation of large molecules and production of free radicals accompany end stages of neuronal death all along. Apoptosis is in itself an energy consuming process, in that when the mitochondrial injury is relatively mild and the amount of ATP produced is enough for the energy consumption of apoptotic process, neuronal apoptosis takes place. On the contrary, if mitochondrial function is seriously injured and ATPs produced drastically exhausted to the extent that is not adequate for initiation of apoptosis, then neuronal necrosis will have taken place. Therefore, biochemical and morphological features of apoptosis and necrosis coexist in the process of neuronal death post-SE, which could be depended on the severity of excitatory injuries and the mitochondrial's functional status. A previous study reported that neuronal apoptosis occurs when the depletion of ATPs in neurons counts less than 70%, whereas neuronal necrosis occurs when the depletion of ATPs in neurons reaches above 70% (Ankarcrona M *et al*., 1995). What also needs to be mentioned is the neurons survived in SE-induced hippocampal injury. This part of neurons, together with neurons staged into apoptosis and necrosis, forms the whole population of neurons in hippocampus. As a result, the number of surviving neurons can be used as a criterion for evaluating the severity of hippocampal injuries and effects of intervention such as quercetin treatment toward epilepsy.

Our results confirmed expression alteration of XIAP and caspase-3 protein in rat hippocampal CA3 region post-SE along with a change in the number of apoptotic and surviving neurons. We demonstrated an expressional alteration of XIAP post-SE, just as a previous study had reported the XIAP expression change in limbic epilepsy rat model induced by kainic acid (Li *et al*., 2004). In our result, the XIAP expression had undergone alterations in amount and space as detected by immunohistochemistry, yet western blot analysis hadn't detected this change as there was no statistic significance between the control and experiment groups, though an incremental trend of the XIAP expression at several time points post-SE is shown in our western blot results (Fig. 3A). The lack of statistical significance in XIAP expression increment as shown in western blot results does not cover the fact that the XIAP expression has indeed undergone an increment in post-SE hippocampus, in that although XIAP can inhibit caspases, XIAP itself can undergo caspase-mediated cleavage to allow the programmed cell death to continue when sufficient activation levels of caspases are reached (Siegelin M *et al*., 2005).

As can be shown in our research results, the early expressional change of XIAP suggested that its up-regulation could be a stress response, which may serve as a brain self-protection mechanism to counteract hippocampal neuronal apoptosis. What needs to be mentioned is that anti-apoptotic capacity of XIAP is associated with its ability to inhibit the activated caspase-3 protein expression (Holcik *et al*., 2001). The devoid of XIAP results into increased neuronal apoptosis after brain injury (West *et al*., 2009). As a result, XIAP could be an exclusive endogenous repressor of caspase cascades. Furthermore, the caspase-3 protein is a key effector for apoptotic cell death. SE can activate apoptotic signaling pathway and lead to an activation of the caspase-3 protein (Weise *et al*., 2005). The over-expression of XIAP could suppress the expression of caspase-3 protein, which would ultimately reduce the extent of apoptotic signaling pathway activation and keep neuronal injuries to a milder extent. Under this premise, we studied quercetin's effects on the expression of XIAP and caspase-3 protein in SE rat model and found that quercetin could lead to an over-expression of XIAP post-SE and a down-expression of the caspase-3 protein, resulting in

hippocampal CA3 region a reduction of apoptotic neurons and an increase of surviving neurons. We deduce that the neuroprotective effects of quercetin, in addition to its intrinsic anti-oxidative effect, could be at least partly due to the modulation of XIAP expression, as our results had demonstrated an association between quercetin treatment and XIAP expression, which had also provided us some hints on a possible target for anti-epileptic interventional measures. However, further mechanistic studies concerning quercetin treatment and subsequent reduced apoptosis are needed in order to give more sufficient evidence to support this hypothesis.

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