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## Research Report

# Platycodin D and 2''-o-acetyl-polygalacin D2 isolated from *Platycodon grandiflorum* protect ischemia/reperfusion injury in the gerbil hippocampus

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### ABSTRACT

*Platycodi radix* is used as a folk remedy for several conditions. In this study, we investigated the neuroprotective effects of five major extracts; deapioplatycoside E (DPE), platycoside E (PE), platyconic acid A (PA), platycodin D (PD) and 2''-o-acetyl-polygalacin D2 (PD2) isolated from the *P. radix* in the hippocampal CA1 region (CA1) 4 or 10 days after ischemia/reperfusion (I/R). Each extract was administered into gerbils with intraperitoneal injection (5 mg/kg/day) 10 days before ischemic surgery and the gerbils were sacrificed 4 or 10 days after I/R. Fluoro-Jade B (F-J B, a marker for neurodegeneration) positive (\*) neurons increased significantly in the stratum pyramidale of the CA1 region in the vehicle-treated group after I/R. A similar pattern was observed in the DPE-, PE- and PA-treated groups; however, in the PD- and PD2-treated groups, F-J B<sup>+</sup> neurons were small in number. We also observed that activations of astrocytes and microglia in the CA1 region after I/R were blocked by the PD- and PD2 treatments. In addition, we found that Cu,Zn-superoxide dismutase (SOD1) immunoreactivity in the pyramidal layer of the PD- and PD2-treated groups was similar to that of the sham group and COX-2<sup>+</sup> and NF-κB<sup>+</sup> cells were significantly lower in the PD- and PD2-treated group than those in the vehicle-treated group after I/R. These results suggest that PD and PD2 rescue neurons in the CA1 region from an ischemic damage.

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## 1. Introduction

The hippocampus, a region responsible for memory and learning, is particularly vulnerable to ischemic damage (Bian et al., 2007; Lorrío et al., 2009). Transient cerebral ischemia induced by the temporary deprivation of blood flow to the brain results in the insidious delayed degeneration of specific vulnerable neurons within the hippocampal CA1 region (CA1) (Kirino, 1982). So far, there are several possibilities about mechanisms of neurodegeneration induced by an ischemia. One of acceptable mechanisms in ischemic neuronal damage is that cellular events involving reactive oxygen species (ROS) mediated oxidative damage may evoke neurodegeneration (Numagami et al., 1996). Recent studies have confirmed the pivotal roles of oxidative stress and inflammatory response in the pathogenesis of ischemic stroke (Glantz et al., 2005; Xia et al., 2006). ROS have been shown to be one of the earliest and most important components of tissue injury after reperfusion in an ischemic organ (Adibhatla and Hatcher, 2003; Nita et al., 2001; Xia et al., 2006).

In oriental countries, *Platycodi radix* is used as a food and a folk remedy for several conditions. Changkil (CK), which is an aqueous extract from the root of *Platycodon grandiflorum* A. DC, prevents hypercholesterolemia and hyperlipidemia (Kim et al., 1995), inhibits tumor invasion and metastasis (Lee et al., 2006), and induces apoptosis of lung cancer (Park et al., 2005). In addition, CK has potent antioxidative effects such as superoxide radical scavenging activity via xanthine and xanthine oxidase system, and protective effect on tert-butyl hydroperoxide-induced oxidative hepatotoxicity by the inhibition of ROS production (Lee and Jeong, 2002; Lee et al., 2004).

Recently, there is an increasing amount of researches focused on functional foods that offer a positive health benefit beyond basic nutrition and concern complementary and alternative medicine (Sandhu and Heinrich, 2005). However,

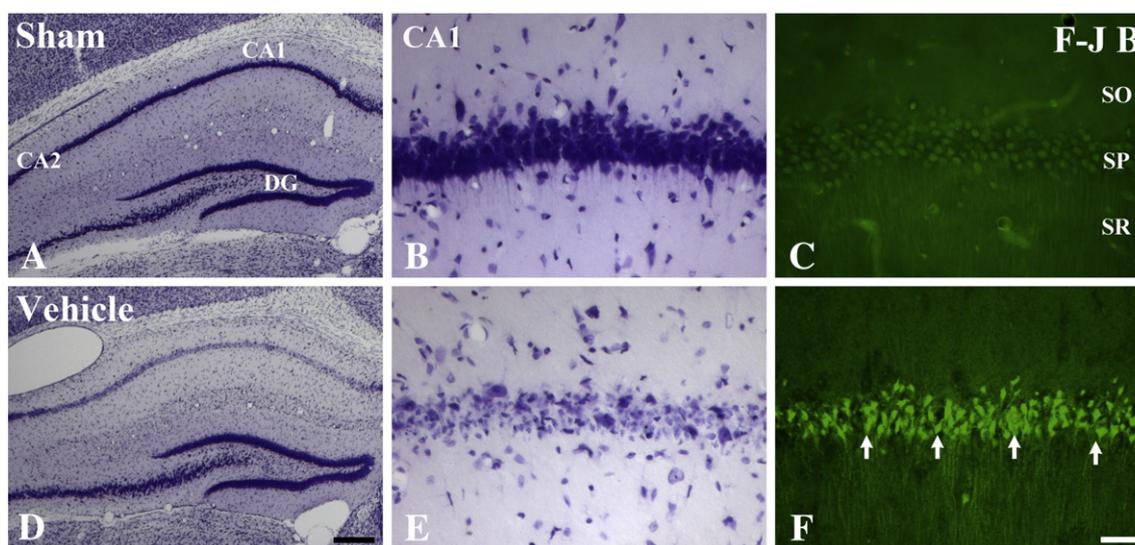
there are no studies about the effects of CK against ischemic damage. In previous studies of our colleagues, a novel triterpenoid saponin, deapioplatycoside E and platyconic acid A were isolated from the root extract of *P. grandiflorum*, together with the known saponins such as platyconic acid A, platycodin D and 2''-o-acetyl-polygalacin D2 (Choi et al., 2008; Kim et al., 2005). In the present study, therefore, we investigated the effects of five extracts isolated from CK against ischemic damage.

## 2. Results

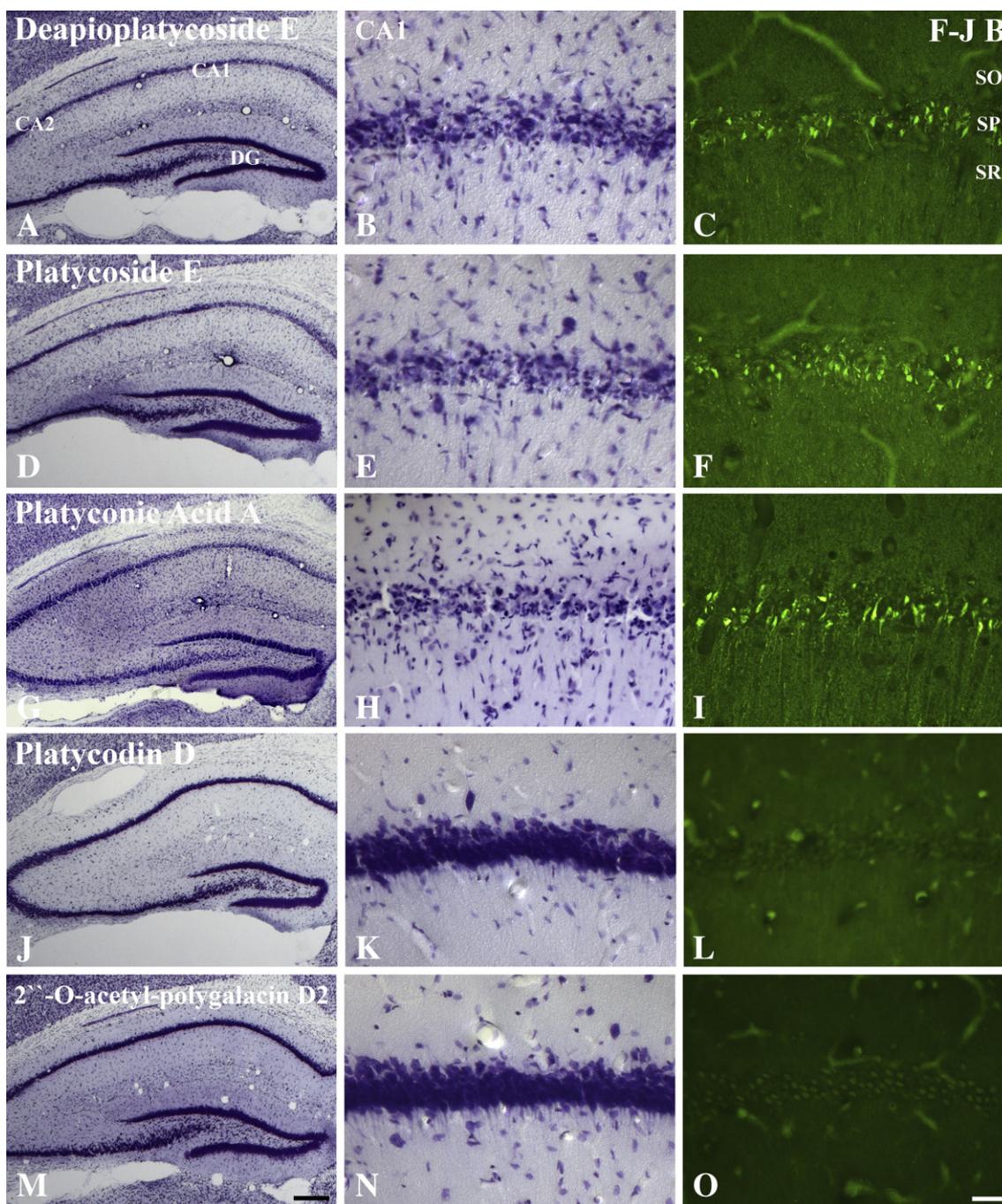
### 2.1. Cresyl violet staining

The neuroprotective effect of DPE, PE, PA, PD and PD2 on pyramidal neurons in the ischemic CA1 region was evaluated by measuring the neuronal number 4 or 10 days after ischemia/reperfusion (Figs. 1, 3 and 4). In the sham group, cresyl violet positive (CV<sup>+</sup>) neurons were observed in all sub-regions of the hippocampus (Figs. 1A and B). However, in the ischemia-operated group, a few CV<sup>+</sup> neurons were detected in the CA1 region 4 or 10 days after I/R, while in the hippocampal CA2/3 region CV<sup>+</sup> neurons were similar to those of the sham group (Figs. 1C, D, and 3A and B). In the ischemic group, 11.3% of pyramidal neurons in the CA1 region were stained with CV: 4 days after ischemia/reperfusion, most of CA1 pyramidal cells were lost due to delayed neuronal death (Fig. 4).

In the DPE, PE and PA-treated groups, a few cresyl violet positive neurons were detected in CA1 region (Figs. 2A, B, D, E, G, H and 4). In the PD and PD2-treated groups, many cresyl violet positive neurons were observed in CA1 region (Figs. 2J, K, M and N): The percentage of survived CA1 pyramidal neurons was 64.2% and 63.1% 4 days after ischemia/reperfusion, respectively (Fig. 4). In these groups, CV<sup>+</sup> neurons were also



**Fig. 1** – CV and F-J B stainings of the gerbil hippocampus in sham (A–C) and ischemia-operated (D–F) groups 4 days after ischemia/reperfusion. In the sham group, CV<sup>+</sup> neurons are detected in all hippocampal sub-regions; however F-J B<sup>+</sup> neurons are not detected. In the ischemia-operated group, a few CV<sup>+</sup> and many F-J B<sup>+</sup> neurons (arrows) are observed in the stratum pyramidale (SP) of the CA1 region due to the delayed neuronal death of pyramidal neurons. SO; Stratum oriens, SR, stratum radiatum. Bar=400 μm (A and D), 50 μm (B, C, E and F).



**Fig. 2** – CV and F-J B stainings of the hippocampus in DPE- (A–C), PE- (D–F), PA- (G–I), PD- (J–L) and PD2- treated (M–O) ischemic groups 4 days after ischemia/reperfusion. In the DPE-, PE- and PA-treated groups, a few CV<sup>+</sup> neurons and many F-J B<sup>+</sup> neurons are observed in the CA1 region. In the PD2- and PD-treated groups, many CV<sup>+</sup> neurons and a few F-J B<sup>+</sup> neurons are detected in the CA1 region, respectively. SO; stratum oriens, SP, stratum pyramidale; SR, stratum radiatum. Bar=400  $\mu$ m (A, D, G, J and M), 50  $\mu$ m (B, C, E, F, H, I, K, L N and O).

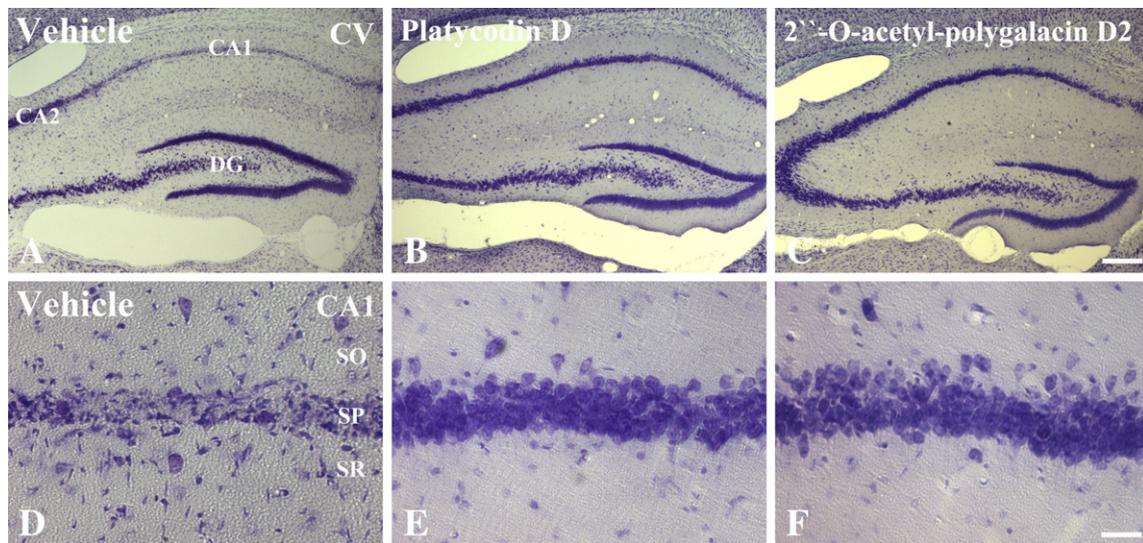
abundant in the CA1 region 10 days after ischemia/reperfusion (Figs. 3C–F): 53.4% and 51.7% of CA1 pyramidal neurons were positive to CV (Fig. 4).

## 2.2. F-J B staining

In the sham group, Fluoro-Jade B positive (F-J B<sup>+</sup>) neurons were not detected in the CA1 region (Fig. 1C). However, in the

vehicle-treated group, the number of F-J B<sup>+</sup> neurons was significantly increased (Figs. 1F and 4).

In the DPE-, PE- and PA-treated groups, F-J B<sup>+</sup> neurons in the CA1 region were numerous like the vehicle-treated group (Figs. 2C, F and I). In the PD- and PD2-treated groups, however, a few F-J B<sup>+</sup> neurons in the CA1 region were observed in the stratum pyramidale of the ischemic CA1 region (Figs. 2L and O).

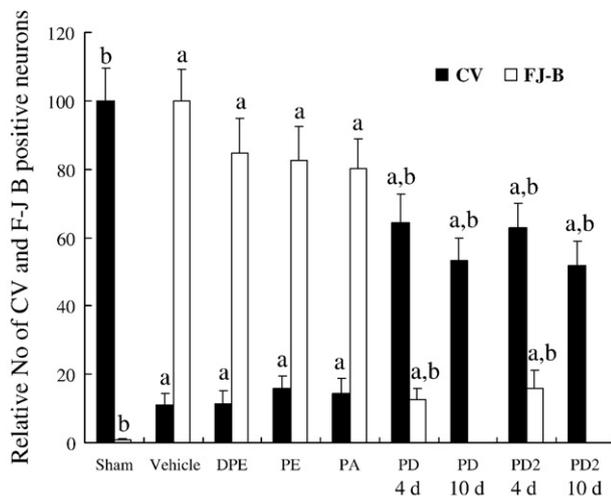


**Fig. 3 – CV staining of the gerbil hippocampus in vehicle- (A and D), PD- (B and E) and PD2-treated (C and F) ischemic groups 10 days after ischemia/reperfusion. Abundant CV<sup>+</sup> neurons are observed in the hippocampal CA1 region in the PD- and PD2-treated groups. SO; stratum oriens, SP, stratum pyramidale; SR, stratum radiatum. Bar=400 μm (A–C), 50 μm (D–F).**

### 2.3. Effects on reactive gliosis

In the sham group, glial fibrillary acidic protein positive (GFAP<sup>+</sup>) astrocytes were threaded in structure, which is the resting form of astrocytes (Fig. 5A). In the vehicle-treated ischemic group, GFAP immunoreactivity was sig-

nificantly increased (Fig. 5H) and GFAP<sup>+</sup> astrocytes showed punctuated cytoplasm 4 days after ischemia/reperfusion, which is the activated form of astrocytes (Fig. 5B). In the DPE-, PE- and PA-treated groups, activated astrocytes were abundant (Figs. 5C–E). In the PD- and PD2-treated groups, many resting forms of astrocytes were detected (Figs. 5F and G).

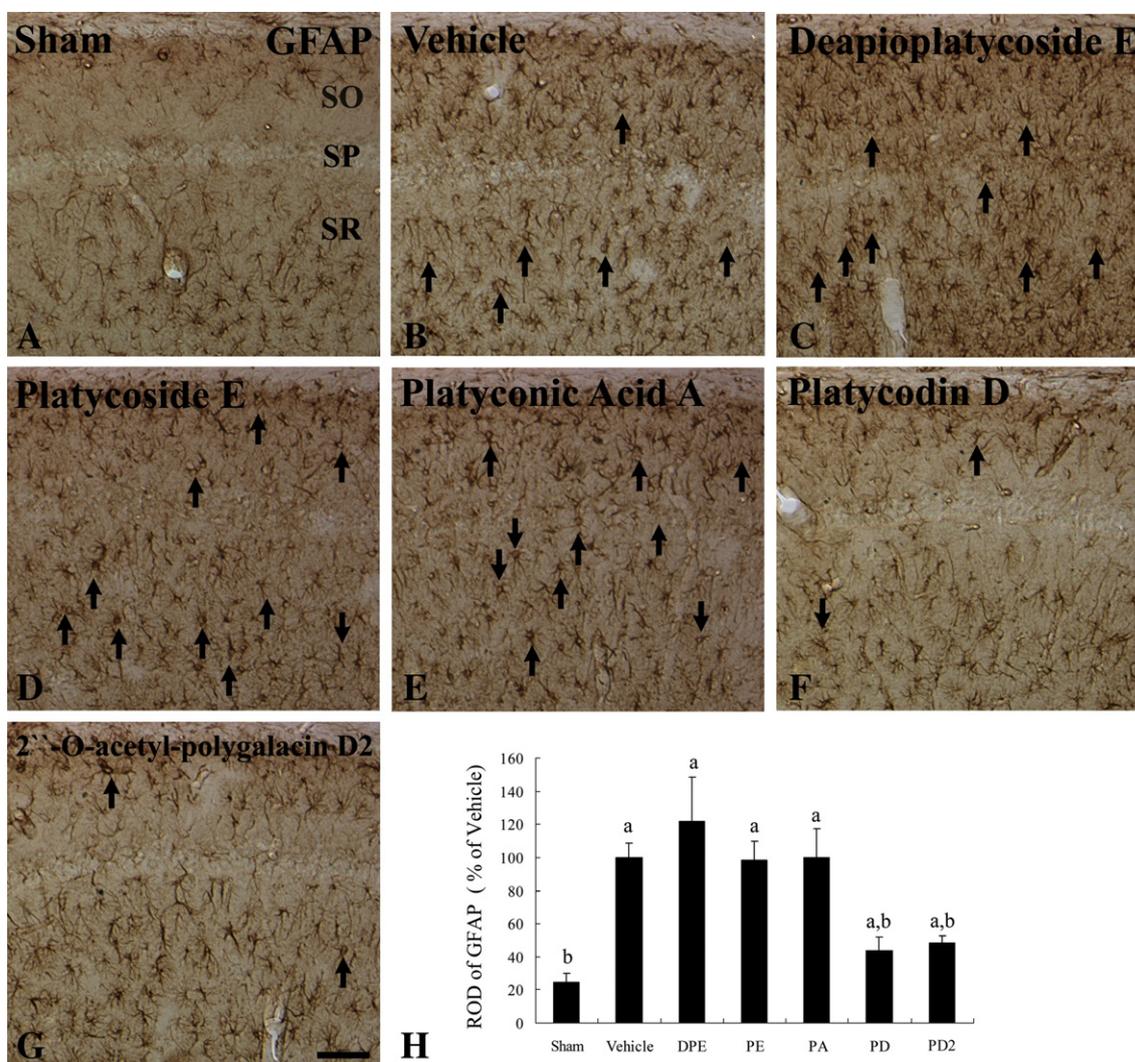


**Fig. 4 – Relative numeric analysis (No) of CV<sup>+</sup> and F-J B<sup>+</sup> CA1 pyramidal neurons. In the PD- and PD2-treated ischemic groups, about 64% and 63% of pyramidal neurons are survived 4 days after ischemia/reperfusion, respectively. Ten days after ischemia/reperfusion, about 53% and 52% pyramidal neurons are survived in the PD- and PD2-treated group, respectively. In the vehicle-, DPE-, PE-, PA-treated group, F-J B<sup>+</sup> significantly increased 4 days after ischemia/reperfusion. (<sup>a</sup>P<0.05, significantly different from the sham group; <sup>b</sup>P<0.05, significantly different from the vehicle-treated group). The bars indicate the means ± SEM.**

Ionized calcium-binding adapter molecule 1 positive (Iba-1<sup>+</sup>) microglia in the sham group had fine processes with web-like networks (Fig. 6A). In the vehicle-treated group, the cytoplasm and processes of microglia were hypertrophied 4 days after ischemia/reperfusion (Fig. 6B) and Iba-1 immunoreactivity significantly increased (Fig. 6H). In the DPE-, PE- and PA-treated groups, microglia were also activated (Figs. 6C–E). In these groups, Iba-1 immunoreactivity was slightly increased compared to that of the vehicle-treated group, respectively (Fig. 6H). In the PD- and PD2-treated groups, many resting form of microglia were detected (Figs. 6F and G), and Iba-1 immunoreactivity was similar to that of the sham group, respectively (Fig. 6H).

### 2.4. Effects on SOD1 immunoreactivity

Cu,Zn-superoxide dismutase (SOD1) immunoreactivity was investigated in the ischemic CA1 region whether DPE, PE, PA, PD or PD2 shows antioxidative effects on ischemic pyramidal neurons. In the sham group, SOD1 immunoreactivity was mainly detected in the stratum pyramidale of the CA1 region (Fig. 7A). In the vehicle-treated group, some SOD1<sup>+</sup> cells were found in the pyramidal layer of hippocampal CA1 region (Fig. 7B). In the DPE-, PE- and PA-treated groups, SOD1 immunoreactivity was markedly decreased in the pyramidal layer compared to that of the sham group (Figs. 7C–E and H). In the PD- and PD2-treated groups, SOD1 immunoreactivity in the pyramidal layer was similar to that of the sham group (Figs. 7F–H).



**Fig. 5** – GFAP immunohistochemistry in the CA1 region in sham (A), vehicle- (B), DPE- (C), PE- (D), PA- (E), PD- (F) and PD2-treated (G) groups 4 days after ischemia/reperfusion. Arrows indicate the activated astrocytes which have a punctuated cytoplasm. In the vehicle-treated group, GFAP<sup>+</sup> astrocytes are activated in the CA1 region. In the PD2- and PD-treated groups, the astrogliosis is markedly decreased. SO; stratum oriens, SP, stratum pyramidale; SR, stratum radiatum. Bar=200  $\mu$ m. H: Relative optical density (ROD) as % of GFAP immunoreactivity in the sham, vehicle-, DPE-, PE-, PA-, PD- and PD2-treated groups. (<sup>a</sup> $P < 0.05$ , significantly different from the sham group; <sup>b</sup> $P < 0.05$ , significantly different from the vehicle-treated group). The bars indicate the means  $\pm$  SD.

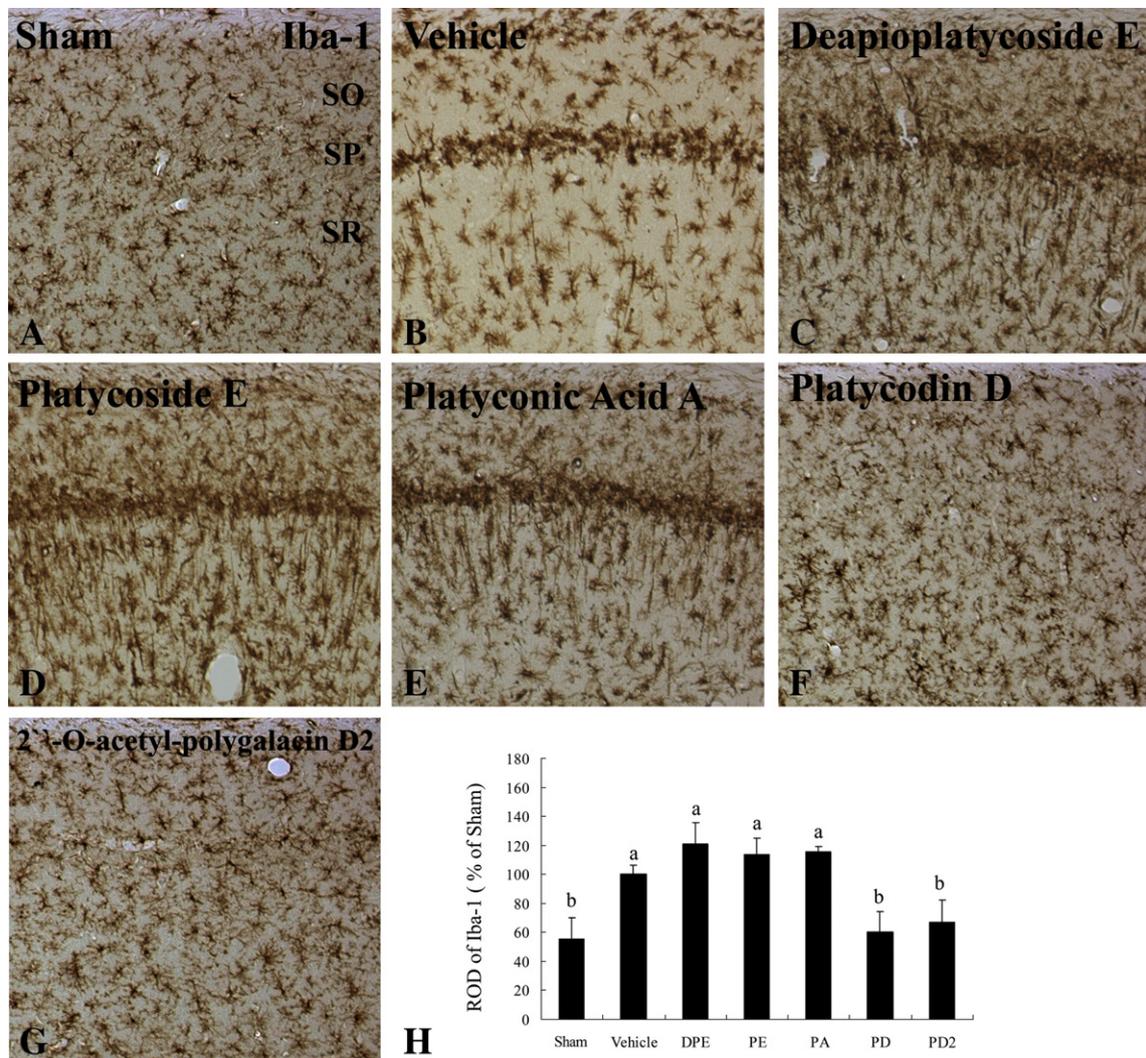
### 2.5. Effects on COX-2 immunoreactivity

In the sham group, a few cyclooxygenase-2 positive (COX-2<sup>+</sup>) cells were observed in the CA1 region (Fig. 8A). However, in the vehicle-treated group, many COX-2<sup>+</sup> cells were detected in the CA1 region (Fig. 8B). In the DPE-, PE- and PA-treated groups, many COX-2<sup>+</sup> cells were observed in the CA1 region and similar to the vehicle-treated group (Figs. 8C–E). In these groups, some COX-2<sup>+</sup> cells were also detected in vasculatures. However, in the PD- and PD2-treated groups, a few COX-2<sup>+</sup> cells were detected in the CA1 region (Figs. 8F and G). COX-2 immunoreactivity in the DPE-, PE- and PA-treated group were similar to that of the vehicle-treated group, however COX-2 immunoreactivity in the PD- and PD2-treated groups was significantly

decreased compared to that of the vehicle-treated group (Fig. 8H).

### 2.6. Effects on NF- $\kappa$ B immunoreactivity

In the sham group, very few nuclear factor kappa B positive (NF- $\kappa$ B<sup>+</sup>) cells were observed in the CA1 region of the hippocampus (Fig. 9A). However, in the ischemia-operated group, many NF- $\kappa$ B<sup>+</sup> cells were detected in the CA1 region (Fig. 9B). In the DPE-, PE- and PA-treated groups, many NF- $\kappa$ B<sup>+</sup> cells were observed and similar to the ischemia-operated group in the CA1 region (Figs. 9C–E). Whereas, in the PD- and PD2-treated groups, a few NF- $\kappa$ B<sup>+</sup> cells were detected in the CA1 region of the hippocampus (Figs. 9F and G). NF- $\kappa$ B immunoreactivity in the DPE-, PE- and PA-treated group was



**Fig. 6** – Iba-1 immunohistochemistry in the CA1 region in sham (A), vehicle- (B), DPE- (C), PE- (D), PA- (E), PD- (F) and PD2-treated (G) groups 4 days after ischemia/reperfusion. In the vehicle-treated group, Iba-1<sup>+</sup> microglia are activated in the CA1 region. In the PD2- and PD-treated groups, the microgliosis is markedly decreased. SO; stratum oriens, SP, stratum pyramidale; SR, stratum radiatum. Bar=200  $\mu$ m. H: Relative optical density (ROD) as % of Iba-1 immunoreactivity in the sham, vehicle-, DPE-, PE-, PA-, PD- and PD2-treated groups (<sup>a</sup>P<0.05, significantly different from the sham group; <sup>b</sup>P<0.05, significantly different from the vehicle-treated group). The bars indicate the means  $\pm$  SD.

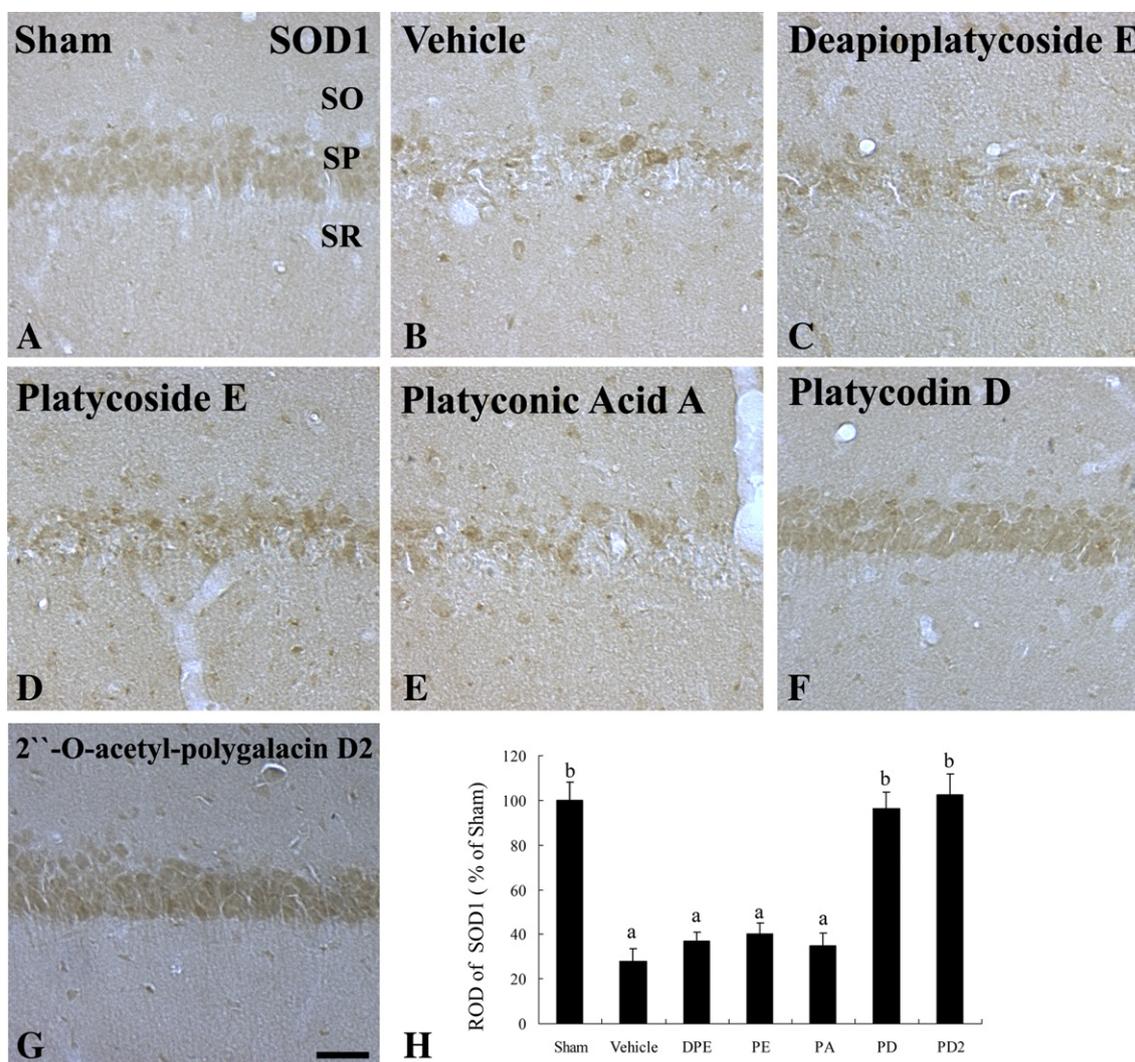
similar to that of the vehicle group, however COX-2 immunoreactivities in the PD- and PD2-treated groups were significantly lower than that of the vehicle-treated group (Fig. 9H).

### 3. Discussion

In this study, we used five components isolated from the CK, and tried to observe the neuroprotective effect of each components against ischemic damage using CV and F-J B histochemistry: We did not observe any neuroprotective effects of DPE, PE and PA. However, PD and PD2 potentially protected CA1 pyramidal neurons from the ischemic damage when we examined 4 or 10 days after I/R. This is the first report in my knowledge that extracts

from the CK have neuroprotective effects against ischemic damage when they were applied via intraperitoneal treatment.

In this study, we also observed that PD and PD2 inhibited the glial activation in the hippocampal CA1 region after ischemia/reperfusion. The hallmark of astroglial response to brain injury is an increase in the level of expression of GFAP (Kato et al., 1994). In the present study, DPE, PE and PA did not decrease the activation of astrocytes in the CA1 region after ischemia/reperfusion. The reactive astrocytes may form the glial scar and help to wall off area of tissue necrosis (Eddleston and Mucke, 1993). However, PD and PD2 treatments significantly reduced the activation of astrocytes in the CA1 region 4 days after ischemia/reperfusion. This result indicates that the neuronal damage is not severe in these groups.

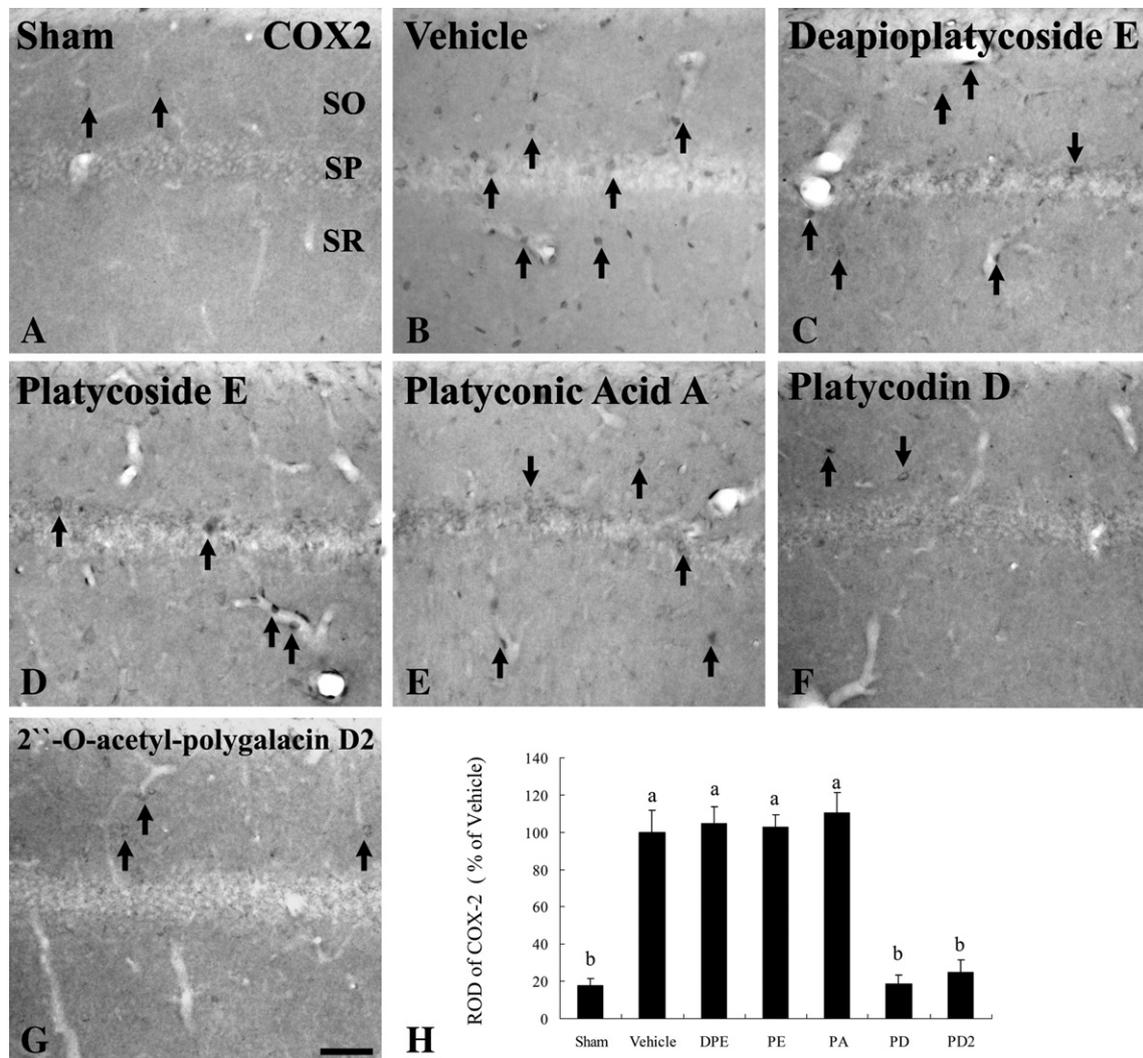


**Fig. 7 – SOD1 immunohistochemistry in the CA1 region in sham (A), vehicle- (B), DPE- (C), PE- (D), PA- (E), PD- (F) and PD2-treated (G) groups 4 days after ischemia/reperfusion. A few SOD1<sup>+</sup> pyramidal cells are observed 4 days after ischemia/reperfusion in the vehicle-, DPE-, PE- and PA-treated groups (B–E). In the PD- and PD2-treated groups, abundant SOD1<sup>+</sup> pyramidal cells are observed in the CA1 region (F and G). SO; stratum oriens, SP, stratum pyramidale; SR, stratum radiatum. Bar = 50  $\mu$ m. H: Relative optical density (ROD) as % of SOD1 immunoreactivity in the sham, vehicle-, DPE-, PE-, PA-, PD- and PD2-treated groups (<sup>a</sup> $P < 0.05$ , significantly different from the sham group; <sup>b</sup> $P < 0.05$ , significantly different from the vehicle-treated group). The bars indicate the means  $\pm$  SD.**

In the present study, the activation of Iba-1<sup>+</sup> microglia was found in the CA1 region of the vehicle-treated group 4 days after ischemia/reperfusion. It was reported that microglia modulated degenerative and regenerative functions in the brain and that microglial reactions appeared to occur early and to be a sensitive and reliable marker for the occurrence of ischemic neuronal damage (Gehrmann et al., 1992). We previously observed that Iba-1<sup>+</sup> microglia were aggregated in the stratum pyramidale of the CA1 region, and their Iba-1 immunoreactivity was very strong 4 days after ischemia/reperfusion (Hwang et al., 2006). In this study, the administration of PD and PD2 isolated from CK blocked the activation and aggregation of microglia in the stratum pyramidale.

Activated microglia produce various neurotoxic factors including cytokines and ROS which have been shown to

trigger neuronal death (Banati et al., 1993; Colton et al., 1994; Greenlund et al., 1995; Palluy and Rigaud, 1996). ROS, such as hydroxyl radical and superoxide anion, are increased after I/R (Hyakkoku et al., 2009; Keles et al., 2008; Won et al., 1999). In the present study, a high level of SOD1 immunoreactivity was observed in the CA1 region of the PD- and PD2-treated groups compared to that of the vehicle-treated group. SOD1 is a specific scavenger of superoxide anion to hydrogen peroxide and molecular oxygen, using hydrogen ions as co-factors (Fridovich, 1995). Transient increase of SOD1 reduces cytokine release from cultured microglia (Chang et al., 2001). In addition, the transduction of SOD1 into the hippocampus reduced neuronal death in ischemic damage (Hwang et al., 2005; Manni and Oury, 2007). Our present results indicate that PD and/or PD2 may alleviate ischemic neuronal damage via antioxidative function.

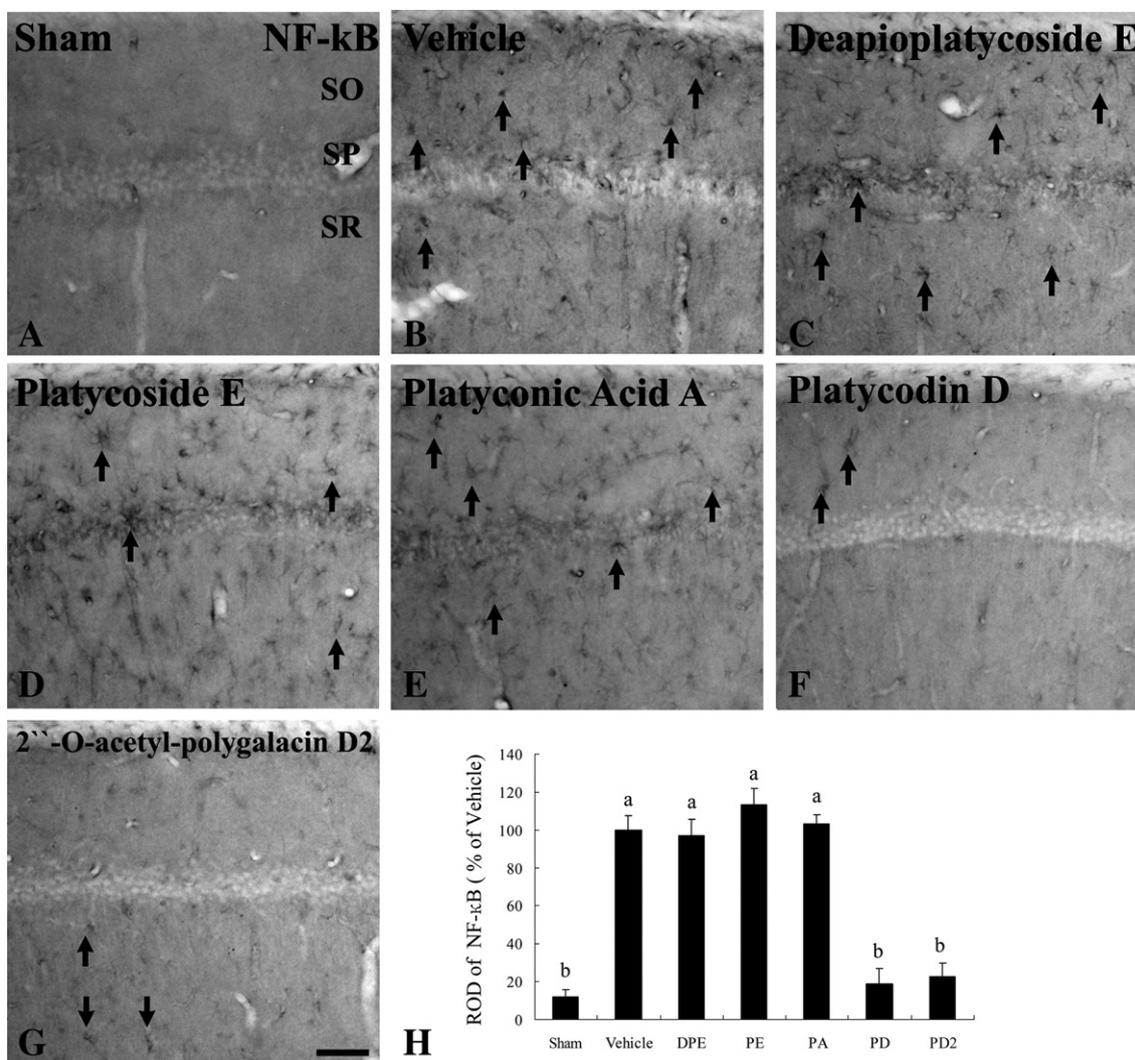


**Fig. 8** – COX-2 immunohistochemistry in the CA1 region in sham (A), vehicle- (B), DPE- (C), PE- (D), PA- (E), PD- (F) and PD2-treated (G) groups 4 days after ischemia/reperfusion. Arrows indicate COX-2<sup>+</sup> cells in each experimental group. In the vehicle-treated group, many COX-2<sup>+</sup> cells (arrows) are observed in the CA1 region. In the DPE-, PE- and PA-treated groups, many COX-2<sup>+</sup> cells are observed in the CA1 region. In the PD3- and PD-treated groups, a few COX-2<sup>+</sup> cells are observed in the CA1 region. SO; stratum oriens, SP, stratum pyramidale; SR, stratum radiatum. Bar=200  $\mu$ m. H: Relative optical density (ROD) as % of COX-2 immunoreactivity in the sham, vehicle-, DPE-, PE-, PA-, PD- and PD2-treated groups (<sup>a</sup> $P < 0.05$ , significantly different from the sham group; <sup>b</sup> $P < 0.05$ , significantly different from the vehicle-treated group). The bars indicate the means  $\pm$  SD.

There are many reports that COX-2 activation causes the over-production of prostaglandins, which plays a key role in the pathophysiology of inflammatory conditions (Jin et al., 2007; Seibert et al., 1994; Singal et al., 2004). NF- $\kappa$ B is a transcription factor that is a key in amplifying the inflammatory response involved in stroke pathophysiology. Increased NF- $\kappa$ B activation has been demonstrated in human stroke (Nurmi et al., 2004a; Terai et al., 1996) and experimental models of brain ischemia (Gabriel et al., 1999; Hill et al., 2001; Ji et al., 2008; Nurmi et al., 2004a). We investigated the anti-inflammatory effects of PD and PD2 because administrations of these potentially blocked the microglial activation. Immunoreactivities of NF- $\kappa$ B<sup>+</sup> and COX-2<sup>+</sup> cells in the CA1 region of the DPE, PE and PA-treated ischemic groups were similar to those in the vehicle-treated group, however immunoreactivities of NF- $\kappa$ B<sup>+</sup> and COX-2<sup>+</sup> cells were much lower in the PD and

PD2-treated groups than those in the vehicle-treated group. This result is supported by previous studies showing that anti-inflammatory drugs prevented neuronal death from an ischemic damage (Ahn et al., 2005; Nurmi et al., 2004b). Ahn et al. (2005) reported in inflammation models that CK and its saponin fraction had an anti-inflammatory activity and reduced COX-2 expression levels by inhibiting the transcription factor, NF- $\kappa$ B, as well as prostaglandin E<sub>2</sub> production *in vitro* in lipopolysaccharide-stimulated mouse macrophage. In addition, CK saponin inhibited carrageenan-induced acute inflammation and the production of a variety of proinflammatory mediators *in vivo* (Kim et al., 2006).

In conclusion, PD and PD2 treatments protect CA1 pyramidal neurons from an ischemic damage and the PD and PD2 treatments block the activation of glial cells. In addition, PD and PD2 treatments significantly reduce the



**Fig. 9** – NF- $\kappa$ B immunohistochemistry in the CA1 in sham (A), vehicle- (B), DPE- (C), PE- (D), PA- (E), PD- (F) and PD2-treated (G) groups 4 days after ischemia/reperfusion. Arrows indicate NF- $\kappa$ B<sup>+</sup> cells in each experimental group. In the vehicle-treated group, many NF- $\kappa$ B<sup>+</sup> cells are observed in the CA1 region. In the DPE-, PE- and PA-treated groups, many NF- $\kappa$ B<sup>+</sup> cells are observed in the CA1 region. In the PD2- and PD-treated groups, a few NF- $\kappa$ B<sup>+</sup> cells are observed in the CA1 region. SO; stratum oriens, SP, stratum pyramidale; SR, stratum radiatum. Bar = 200  $\mu$ m. H: Relative optical density (ROD) as % of NF- $\kappa$ B immunoreactivity cells in the sham, vehicle-, DPE-, PE-, PA-, PD- and PD3-treated groups (<sup>a</sup> $P < 0.05$ , significantly different from the sham group; <sup>b</sup> $P < 0.05$ , significantly different from the vehicle-treated group). The bars indicate the means  $\pm$  SD.

neuro-inflammation induced by ischemia/reperfusion in the hippocampal CA1 region.

#### 4. Experimental procedures

##### 4.1. Preparation of extracts of CK

Dried roots of *P. grandiflorum* (5 kg) were extracted three times with methanol at room temperature for 7 days. Concentration of the solvent gave a brown syrupy extract (1.4 kg) which was suspended in water and then partitioned successively with ethyl acetate (63 g) and *n*-butanol (130 g). The *n*-butanol layer was suspended in H<sub>2</sub>O (2 L) and poured onto a Diaion HP-20 column ( $\phi = 5.0 \times 100$  cm), which was stabilized with H<sub>2</sub>O. The column was washed with H<sub>2</sub>O (2 L) and then eluted with MeOH

(5 L). Five fractions; deapioplatycoside E (DPE), platycoside E (PE), platyconic acid A (PA), platycodin D (PD) and 2''-o-acetyl-polygalacin D2 (PD2) were prepared from the extract of *P. grandiflorum* for experimental procedures using our method (Choi et al., 2008).

##### 4.2. Experimental animals and treatment with extracts isolated from CK

Male Mongolian gerbils (*Meriones unguiculatus*) (6 months of age) were obtained from the Experimental Animal Center, Hallym University, Chuncheon, South Korea. The procedures for handling and caring for animals adhered to the guidelines that are in compliance with the current international laws and policies (NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85-23, 1985, revised 1996). All

of the experiments were conducted minimizing the number of animals used and the suffering caused by the procedures used in the present study.

The gerbils were divided into 7 groups ( $n=7$  per each group): sham, vehicle (saline)-, DPE-, PE-, PA-, PD-, and PD2-treated groups. These extracts were administered into gerbils with 0.5 ml intraperitoneal injection (5 mg/kg/day) for 10 days before an ischemic surgery: The last was administered 30 min before the ischemic surgery. The animals were sacrificed 4 days or 10 days after the ischemic surgery.

#### 4.3. Induction of transient forebrain ischemia

Animals were placed under general anesthesia with a mixture of 2.5% isoflurane (Baxter, Deerfield, IL) in 33% oxygen and 67% nitrous oxide. A midline ventral incision was made in the neck and both common carotid arteries were isolated, freed of nerve fibers, and occluded using non-traumatic aneurysm clips. Complete interruption of blood flow was confirmed by observing the central artery in eyeballs using an ophthalmoscope. After 5 min of occlusion, the aneurysm clips were removed from the common carotid arteries. Restoration of blood flow (reperfusion) was observed directly under the ophthalmoscope. We maintained the body (rectal) temperature under free-regulating or normothermic ( $37 \pm 0.5$  °C) conditions with a rectal temperature probe (TR-100; Fine Science Tools, Foster City, CA) and thermometric blanket before, during the surgery and after the surgery until the animals fully recovered from anesthesia. Sham-operated animals were subjected to the same surgical procedures except that the common carotid arteries were not occluded (Hwang et al., 2007).

#### 4.4. Tissue processing for histology

For histology, all experimental animals were anesthetized with pentobarbital sodium and perfused transcardially with 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in 0.1 M PB (pH 7.4) at the 4 days and 10 days after the ischemic surgery. Brains were removed and postfixed in the same fixative for 6 h. The brain tissues were cryoprotected by infiltration with 30% sucrose overnight. Thereafter frozen tissues were serially sectioned on a cryostat (Leica, Wetzlar, Germany) into 30- $\mu$ m coronal sections, and they were then collected into six-well plates containing PBS.

#### 4.5. Cresyl violet staining

Cresyl violet (CV) staining was conducted 4 and 10 days after the ischemic surgery. In brief, the sections were mounted on gelatin-coated microscopy slides. Cresyl violet acetate (Sigma, St. Louis, MO) was dissolved at 1.0% ( $w/v$ ) in distilled water, and glacial acetic acid (Sigma) was added to this solution. Before and after staining for 2 min at room temperature, the sections were washed twice in distilled water. The fixed brain tissues were dehydrated by immersing for 2 h in 50%, 70%, 80%, 90%, 95% and 100% ethanol baths in succession at room temperature. After dehydration, the sections were mounted with Canada Balsam (Kanto, Tokyo, Japan).

#### 4.6. Fluoro-Jade B histofluorescence

To examine effects of DPE, PE, PA, PD and PD2 against ischemic damage, the sections were stained by Fluoro-Jade B (F-J B), a marker for neurodegeneration, histofluorescence (Candelario-Jalil et al., 2003). In brief, the sections were mounted on gelatin-coated microscopy slides. They were then first immersed in a solution containing 1% sodium hydroxide in 80% alcohol, and followed in 70% alcohol. They were then transferred to a solution of 0.06% potassium permanganate, and transferred to a 0.0004% F-J B (Histochem, Jefferson, AR) staining solution. After washing, the sections were placed on a slide warmer (approximately 50 °C), and then examined using an epifluorescent microscope (Carl Zeiss, Göttingen, Germany) with blue (450–490 nm) excitation light and a barrier filter. With this method, neurons that undergo degeneration brightly fluoresce in comparison to the background (Schmued and Hopkins, 2000).

#### 4.7. Immunohistochemistry for GFAP, Iba-1, SOD1, COX-2 and NF- $\kappa$ B

To confirm the reactive gliosis, and anti-inflammatory effects of DPE, PE, PA, PD and PD2 in the CA1 of the hippocampus after ischemia/reperfusion, immunohistochemistry was processed under the same conditions 4 days after the surgery. The sections were sequentially treated with 0.3% hydrogen peroxide ( $H_2O_2$ ) in PBS for 30 min and 10% normal goat serum in 0.05 M PBS for 30 min. They were next incubated with rabbit anti-GFAP (diluted 1:1000, Chemicon International, Temecula, CA) for astrocytes and rabbit anti-Iba-1 (diluted 1:500, Wako, Osaka, Japan) for microglia, mouse anti-SOD1 (diluted 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA) for neuronal protection, rabbit anti-COX-2 (1:200, Cayman, Ann Arbor, MI) and rabbit anti-NF- $\kappa$ B (diluted 1:2000, AbSerotec, Brentwood, NH) for anti-inflammation overnight at 4 °C, and subsequently exposed to biotinylated goat anti-rabbit IgG (diluted 1:200, Vector, Burlingame, CA), goat anti-mouse IgG and avidin-biotin complex kit. They were then visualized by staining with 3,3'-diaminobenzidine (Sigma) in 0.1 M Tris-HCl buffer (pH 7.2) and mounted on gelatin-coated slides. After dehydration the sections were mounted with Canada Balsam (Kanto).

Negative controls for all immunohistochemical experiments were generated by incubation in rabbit IgG at the same concentration as the primary antibodies. The negative control resulted in the absence of immunoreactivity in all structures.

#### 4.8. Quantification of data and statistical analysis

All measurements were performed in order to ensure objectivity in blind conditions, by two observers for each experiment, carrying out the measures of control and experimental samples under the same conditions. To elucidate the effects of DPE, PE, PA, PD and PD2 against ischemic damage after I/R, neuronal number assessment was performed using an image analyzing system equipped with a computer-based CCD camera (software: Optimas 6.5, CyberMetrics, Scottsdale, AZ). CV<sup>+</sup> and F-J B<sup>+</sup> neurons were counted in a 250  $\times$  250  $\mu$ m square, applied approximately at the center of the CA1 region in the

stratum pyramidale. Cell counts were obtained by averaging the counts from 15 sections taken from each animal. The number of CV<sup>+</sup> and F-J B<sup>+</sup> neurons was compared to those of the sham-operated group (Hwang et al., 2007).

In order to quantitatively analyze GFAP, Iba-1, SOD1, COX-2 and NF- $\kappa$ B immunoreactivity, the corresponding areas of the hippocampal CA1 region were measured from 15 sections per animal. Images of all GFAP<sup>+</sup>, Iba-1<sup>+</sup>, SOD1<sup>+</sup>, COX-2<sup>+</sup> and NF- $\kappa$ B<sup>+</sup> structures were taken from 3 layers (strata oriens, pyramidale and radiatum in the hippocampus proper) through an AxioM1 light microscope (Carl Zeiss, Germany) equipped with a digital camera (AxioCam, Carl Zeiss) connected to a PC monitor. Images were calibrated into an array of 512×512 pixels corresponding to a tissue area of 140×140  $\mu$ m (40× primary magnification). Each pixel resolution was 256 gray levels. The densities of all GFAP<sup>+</sup>, Iba-1<sup>+</sup>, SOD1<sup>+</sup>, COX-2<sup>+</sup> and NF- $\kappa$ B<sup>+</sup> structures were evaluated on the basis of an optical density (OD), which was obtained after the transformation of the mean gray level using the formula: OD=log (256/mean gray level). The OD of background was taken from areas adjacent to the measured area. After the background density was substrated, a ratio of the optical density of image file was calibrated as % (relative optical density, ROD) using Adobe Photoshop version 8.0 and then analyzed using NIH Image 1.59 software. Data are expressed as the mean±SEM. The data were evaluated by a one-way ANOVA SPSS program, and the means assessed using Duncan's multiple-range test. Statistical significance was considered at  $P < 0.05$ .

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