Cyanidin prevents hippocampal cell death and promotes astrocytosis in kainic acid-induced neurodegeneration

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ABSTRACT
Temporal lobe epilepsy (TLE) is one of the non-communicable diseases characterized by dentate granule cell dispersion (GCD) and the loss of the CA1 and CA3 neurons. This study investigated the effects of cyanidin on neurodegeneration after KA injection. Male Wistar rats were divided into a saline-injected group as a control, KA alone and cyanidin treated group at a dose of 10 mg/kg BW seven days before and after KA injection. The histomorphological analysis revealed that GCD was reduced in the ipsilateral hippocampus of cyanidin treated group when compared with KA alone (P<0.05). There was no neurodegeneration observed in the contralateral hippocampus. In contrast, neuronal degeneration was limited in the ipsilateral CA1 (P<0.01) and the hilar interneurons (P<0.001) of cyanidin treated group when compared with KA alone. However, there was no significant difference in the number of CA3 neurons of the ipsilateral between KA alone and cyanidin treated group. GFAP staining revealed a higher number of reactive astrocytes in the ipsilateral hilus (P<0.001) and molecular layer (P<0.05) of cyanidin treated group when compared with KA alone. Taken together, cyanidin could prevent neurodegeneration and promote astrocyte proliferation as well as mitigated GCD following KA-induced hippocampal injury.

INTRODUCTION
Temporal lobe epilepsy (TLE) is characterized by spontaneous seizures results in neuron loss and granule cell dispersion (GCD) in the hippocampus. Nowadays, antiepileptic drugs are used as the treatment for epilepsy. However, almost half of the patients resist to the drugs (Laxer et al., 2014). Moreover, the present pharmacotherapies produce several side effects (Edie, 2012). A study showed that an imbalance between over-excitatory of glutamate and the less inhibitory activities of the GABAergic system results in over-excitation of the neuron leads to neuronal death. Therefore, the aim of pharmacological therapies is to reduce the effects of glutamatergic overflow that can cause cell death and promote inhibitory activities of the GABAergic system (Aleksey et al., 2019). Kainic acid (KA) binds to glutamate receptors (GluR) results in the increase of intracellular Ca²⁺ followed by activation of Ca²⁺ dependent enzyme (COX2, proteases, kinases, nucleases, phospholipases) that causes excessive free radicals (reactive oxygen species; ROS, reactive nitrogen species; RNS) leading to neuronal necrosis eventually cell death (Xing-Mei et al., 2011).

Cyanidin is an organic compound in the anthocyanin group which belongs to the flavonoids family. It has
Intrahippocampal Injections of KA

KA injection was performed using stereotactic apparatus. The rats were deeply anesthetized with pentobarbital sodium (50 mg/kg, i.p.; Ceva Sante´Animale). They were immobilized on a stereotactic apparatus (RWD, LIFE SCIENCE). The coordinates from bregma areas followed; AP = −3.8 mm; ML = −2 mm; DV = −2 mm to target the dorso-hippocampus according to the stereotactic coordinates of Paxinos and Watson’s rat brain atlas (Paxinos and Watson, 1998). KA (EMD Millipore) is freshly prepared (5 mL of sterile normal saline containing 1 mg of kainate) then 50 mL of KA solution was injected with a 0.5 mL Hamilton syringe. The control group was received the same volume of normal saline. Seven days later, rats were sacrificed by an overdose of pentobarbital sodium (150 mg/kg), followed by transcardial perfusion with 4% paraformaldehyde in 50 mM phosphate-buffered saline (PBS), pH 7.2. Following the perfusion, the brains were washed with PBS then immersed in 20% sucrose in PBS at 4°C for 2 days. Then, sections were cut at a 30 μm thickness on a cryostat microtome (AST 500 semi-automatic cryostat microtome).

Histological Analysis

The brain sections were stained with 0.1% cresyl violet (Sigma-Aldrich) for investigating the morphological change of the hippocampus. Immunohistochemistry was used to detect the change of reactive astrocytes, and the sections were blocked by 10% normal goat serum and 0.3% Triton X-100 in PBS for 90 minutes. Primary antibody, rabbit anti-glial fibrillary acidic protein (GFAP, 1:500, EMD Millipore) was incubated overnight at room temperature. After that, the sections were washed with PBS and incubated with horseradish peroxidase conjugated secondary antibody (1:500, EMD Millipore). 3,3′-diaminobenzidine was used for detection. Hippocampal neurons were counts and GCD was measured from the sections near the injected site. In cresyl violet-stained sections, neuronal loss was quantified in CA1 and CA3 regions of the hippocampus. Pyramidal neurons were assessed in regions of interest (125 μm length, covering the whole width of the layer). Interneurons were counted in the hilus of the dentate gyrus. Reactive astrocytes were counted in the hilus and molecular layer of the dentate gyrus (10,000 μm²). All data were analyzed by the SX view program.

Statistical Analysis

One-way ANOVA, followed by Tukey’s post hoc test, was used to analyze the data. The data were shown as means±standard error of mean (SEM) using GraphPad Prism7 software (* = P<0.05, **P = <0.01, ***P = <0.001).

RESULTS AND DISCUSSION

Effect of cyanidin on GCD after KA injection

The GCD was observed in the hippocampus after KA injection. GCD has been shown to develop in 1 week after administration of KA and clearly obvious after 3 weeks (Suzuki et al., 1995; Thongrong et al., 2016). Seven days after KA injection, GCD was observed in the ipsilateral (Figure 1 B and Figure 1 C). This GCD was significantly more observed in KA alone when compared with cyanidin treated...
group (160,744±6,932 μm² vs. 122,766±5,104 μm²; *P<0.05; Figure 1 D). GCD was not detected in the contralateral hippocampus of KA injected (data not shown) and in saline-injected rats.

**Hippocampus cytoarchitecture in the Nissl staining**

The neurodegeneration in rat hippocampus was demonstrated by counting neuronal number in CA1, CA3 and hilar interneurons. After KA injection, neurodegeneration was prominent in the ipsilateral CA1 (Figure 2 D and Figure 2 E), CA3 (Figure 2 I and Figure 2 J) and hilar (Figure 2 N and Figure 2 O). Our results showed that cyanidin treated rats revealed a significant difference in a number of neurons in the ipsilateral CA1 when compared with KA injection alone (54.17±3.08 vs. 38.83±2.44; **P<0.01; Figure 2 P). In contrast, the ipsilateral CA3 showed no significant difference in the number of neurons (Figure 2 Q). Hilar interneurons were protected by cyanidin in the ipsilateral (21±0.97 vs.13.17±0.88; ***P<0.001; Figure 2 O and Figure 2 R). However, in the contralateral, there were no significant differences in the number of neurons in CA1, CA3 of and interneurons in the hilus (Figure 2).

**Alterations in reactive astrocytes**

Reactive astrocytes are increased in epilepsy and play a role to promote recovery (Devinsky et al., 2013). Seven days after KA injection, there was the increased in the number of reactive astrocytes in the ipsilateral hilus (Figure 3 D and Figure 3 E) and molecular subfields of dentate gyrus (Figure 3 I and Figure 3 J). The number of reactive astrocytes in the ipsilateral hilus and molecular layer was markedly increased in both KA alone and KA with cyanidin treated groups (Figure 3 K and Figure 3 L). In the ipsilateral hilus, cyanidin treated group showed a higher number of reactive astrocytes when compared with KA injection alone (57.33±2.60 vs. 40.67±2.14; ***P<0.001; Figure 3 K) as well as in the molecular layer (53.33±2.78 vs. 40.67±3.73; *P<0.05; Figure 3 L). However, there was no significant difference in all subfields of the contralateral.

This study demonstrated that cyanidin could prevent KA-induced neurodegeneration in adult hippocampus. However, the mechanism of this effect is not fully understood. Over stimulation of kainate receptors by KA results in high Ca²⁺ influx into the neurons. Calcium ions activated Ca²⁺-dependent enzyme leading to the production of free radicals (ROS and RNS). High levels of free radicals induce mitochondria dysfunction and damage of cell membrane eventually cell death (Xing-Mei et al., 2011). Our results demonstrated that pretreatment with cyanidin 7 days before and post-treatment 7 days after KA injection significantly protected against excitotoxicity after KA injection. As cyanidin is
Figure 2: The injection of saline or KA. Cyanidin prevents cell death in the ipsilateral CA1 (D, E, P; **P<0.01), and hilus (N, O, R; ***P<0.001) but not CA3 (I, J, Q). There are no changes in the contralateral (B, C, G, H, L, M). The data are shown as mean ± SEM (n=6), bar=100 μm.

belonged to the anthocyanin family and contains antioxidant activity, we suggested that the protective effect of cyanidin in KA-induced excitotoxicity is possibly associated with suppression of free radicals. In mouse hippocampal cell line (HT22) and primary prenatal rat hippocampal neurons, anthocyanins have been shown to diminish KA-induced dysregulation of Ca^{2+}, accumulation of ROS and prevent neurodegeneration in the hippocampus (Ullah et al., 2014). The study has demonstrated that ROS has been involved in the seizure-induced neurodegeneration. The production of free radicals induced lipid peroxidation; however, antioxidants such as vitamin E and glutathione have the potential effects to prevent the increasing level of lipid peroxidation and hippocampal cell loss in the development of seizure (Frantseva et al., 2000). TLE is characterized by loss of CA1 and CA3 principal neurons and in the hilar interneurons (Lee et al., 2010). It has been reported that hilar interneurons are sensitive to KA-induced excitotoxicity via increased ROS and RNS (Ueda et al., 2002). In our study, cyanidin treated rats showed a higher number of hilar interneurons compared to the KA injected group after administration of KA suggested the beneficial effects of antioxidants on the lesion model used here. Many studies have reported that consumption of flavonoids such as apigenin, luteolin and genistein can reduce in KA-induced mitochondrial ROS and number of apoptotic neurons in vitro and in vivo and significantly reduce the latency of seizure onset (Avallone et al., 2000; Zhen et al., 2016; Khodamoradi et al., 2016). GCD is associated with hippocampal sclerosis in 50% of epilepsy patients (Kobow et al., 2009). In this study, we demonstrated that GCD was diminished by cyanidin.
Figure 3: KA induced reactive astrocytes in the ipsilateral hilus and molecular layer. Cyanidin displays more reactive astrocytes (arrowheads) in hilus (E, K) and molecular layer (J, L). The data are shown as mean ± SEM (n=6), bar=100 µm. (*P<0.05, ***P<0.001)

Likely, oral administration of morin, a bioflavonoid 1 day before the KA injection, and then administered for 7 more days markedly reduced the KA-induced GCD in the hippocampus by reducing oxidative stress (Yang et al., 2015).

In this experiment, cyanidin promoted a higher number of reactive astrocytes when compared with KA alone. A study in a mouse model showed that reactive astrocytes secret gial derived neurotrophic factor (GDNF) and glutathione, an antioxidant which can protect neuronal death from 6OHDA (Sandhu et al., 2009). The recent studies revealed that astrocytes are an important source of antioxidants in the brain and play a role in cellular defense mechanisms against injury (Ma, 2013). In addition, a study in mice showed that astrocytes can prevent neurons from glutamate-induced excitotoxicity (Rothstein et al., 1996).

CONCLUSIONS

In conclusion, this research provides the effect of cyanidin on KA-induced neurodegeneration, GCD and reactive astrocitosis. Neurodegeneration and GCD are reduced while reactive astrocitosis is clearly enhanced in cyanidin treated rats after administration of KA. So far, many flavonoids have been reported to protect neurodegeneration in the epileptic model suggest that cyanidin, a member of the flavonoid family, could have the potential to become the alternative medicines for developing novel therapeutic strategies for the treatment of epilepsy.

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