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# Geniposide attenuates postischemic long-term potentiation via GluN2A

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**Abstract**: N-Methyl-D-aspartate receptor (NMDAR)-induced antioxidation is a significant cause of neuronal injury after ischemic stroke. In a previous work, we verified the neuroprotective roles of geniposide during tMCAO *in vivo*. However, it remains unknown whether geniposide ameliorates injury to hippocampal neurons during Ischemic Long Term Potentiation (iLTP) induction *in vitro*. After induction of cells oxygen-glucose deprivation or hydrogen peroxide, the protection of geniposide evaluated by MTT assay and electrophysiological tests. In this study, we suggested neuronal cell apoptosis was attenuated by geniposide. Furthermore, field excitatory postsynaptic potentials (fEPSCs) following postischemic LTP were assessed by electrophysiological tests. Finally, we determined that medium and high doses of geniposide attenuated oxidative stress insult and improved iLTP. Importantly, these effects were abolished by cotreatment with geniposide and the GluN2A antagonist NVP. In contrast, the GluN2B inhibitor ifenprodil failed to have an effect. In conclusion, we suggest for the first time that treatment with geniposide can attenuate postischemic LTP induction in a concentration-dependent manner. We infer that GluN2A-containing NMDARs are involved in the neuroprotection induced by geniposide treatment in ischemia.

**Keywords**: Geniposide, NMDAR, GluN2A, ischemic long-term potentiation, antioxidation.

## INTRODUCTION

Stroke is one of the major causes of disability and mortality worldwide. Evidence shows that more than 80% of ischemia is caused by cerebral artery infarction (Hankey 2017). Emerging data have shown that oxidative stress is a significant aspect of brain injury and may be regarded as the triggering factor in neurodegenerative disorders (Li-xia *et al.,* 2009, Luo *et al.,* 2018). These pathological processes may result in neurological deficits after ischemic stroke.

Geniposide is an iridoid glycoside extracted from the fruit of *Gardenia jasminoides Ellis*, which is a main ingredient of various Chinese herbal medicines (Gaire and Prasad 2018, Hou *et al.,* 2008). Geniposide is a cyclic ether terpene glycoside compound that is used to cure some disorders, such as acute conjunctivitis and inflammatory diseases (Pan *et al.,* 2013). Previously, many studies have proven that geniposide can exert various effects in multiple pathological conditions, including antipyretic (Gong *et al.,* 2014), antioxidative (Yin *et al.,* 2010), and antidiabetic effects (Wu *et al.,* 2009). The great significance of geniposide involves both its antioxidative and neuroprotective properties. Studies have shown that geniposide enhances antioxidant capacity via PI3K/Nrf2 signaling in primary hippocampal neurons (Yin *et al.,* 2010). Another report showed that geniposide protects hippocampal slice cultures from oxygen and glucose deprivation-induced neuronal death (Lee *et al.,* 2006). Increasing evidence has proven that N-methyl-D-aspartate receptor (NMDAR) over activation is a key factor in the induction of excitotoxicity. Meanwhile, $Ca^2$+ overload is another factor that triggers cell death (Lai *et al.,* 2011). Selective strategies for restoring the normal status of NMDA receptors and $Ca^2$+ channels seem to be helpful in the postischemic brain (Hardingham 2010, Yao *et al.,* 2012). Two major NMDA receptor subunits, GluN2A and GluN2B, are highly expressed in the hippocampus and participate in different pharmacological and electrophysiological phenomena (Monyer *et al.,* 1994). Ischemic long-term potentiation (iLTP) is triggered by excessive calcium influx and the activation of NMDA glutamate receptors after ischemic stroke (Wang *et al.,* 2015). Our previous work demonstrated that geniposide protects neurons against postischemic neurovascular injury through the activation of GluN2A/AKT/ERK pathways *in vivo* (Huang *et al.,* 2017).

The objective was to investigate the neuroprotective effects of geniposide under OGD conditions in vitro. Studies were also undertaken to explore whether such cytoprotection are involved in the field excitatory postsynaptic potentials (fEPSCs) following OGD damage. However, whether and how geniposide has cytoprotective effects on hippocampal neurons *in vitro* after ischemic stroke have not been clarified.

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We found that geniposide dose-dependently protected neuronal survival in the presence of hydrogen peroxide in PC12 cells. In addition, in slices, geniposide ameliorated impairment in fEPSPs following LTP after ischemia through the GluN2A receptor. These results provide insights into geniposide to advance our understanding of its potential as a treatment target for neurovascular ischemic disorders.

## MATERIALS AND METHODS

### *Drug and reagents*

Geniposide (98% purity) was purchased from Yuanye Shanghai Biotechnology Corp Ltd (China). Hydrogen peroxide ($H_2O_2$), ifenprodil and NVP-AAM077 were purchased from Beyotime Company (China). An Axopatch-200B amplifier (Axon Instruments, USA) and Clampfit 10.2 software (Molecular Devices, USA) were used. All chemical agents were of analytical grade.

### *Animals*

Male Sprague-Dawley rats (180-200g) were purchased from Shanghai Super-B&K Laboratory Animal Corp., Ltd. The animals were housed under a natural day-night cycle with ad libitum access to food and water. The room temperature and relative humidity were maintained at 22±2°C and 58±3%, respectively. All animal studies followed the guidelines of the ethics committee of Wenzhou University.

### *Cell viability assay*

PC12 cells were placed in 6-well plates at a density of $5x10^5$cell/ml. To evaluate the cytoprotective effects of geniposide, PC12 cells were treated with different concentrations of geniposide and $H_2O_2$ for 24h at 37°C. Finally, cell viability was analyzed by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) colorimetric assay. The cells were incubated with MTT (0.5mg/ml final concentration) at 37°C for two hours. Then, they were incubated in fresh complete medium. In the medium, activated cells reduced MTT to purple formazan. Finally, we used a microplate reader to measure the absorbance.

### *Electrophysiology and OGD induction*

Rat coronal slices (350μm thick) were cut with a microtome with a vibrating blade. The slices were incubated with artificial cerebrospinal fluid (ACSF) (34°C, 2.5-3ml/min, bubbled with 95% $O_2$/5% $CO_2$) with the following composition (mmol/l): 126 NaCl, 2.5 KCl, 1.2 $MgCl_2$, 1.2 $NaH_2PO_4$, 2.4 $CaCl_2$, 11glucose and 25 $NaHCO_3$ for one hour. An Axopatch-200B amplifier (Molecular Devices, Palo Alto, CA) was used to record field excitatory postsynaptic potentials (fEPSP) in the hippocampal CA1 region (Chen *et al.,* 2015, Yao *et al.,* 2012). A bipolar tungsten electrode was positioned in the Schaffer collaterals. After 10 min of baseline recording, postischemic LTP (iLTP) was then induced by cotreatment with geniposide and oxygen and glucose deprivation (OGD; glucose was replaced with ACSF and sucrose gassed with 95% $N_2$ and 5% $CO_2$) for another 10 min. The fEPSP initial slope value/control slope value was determined by Clampfit 10.2 software (Molecular Devices, USA). The mean response value was obtained and analyzed by pClamp 10.2 software (Molecular Devices, USA). All experiments were performed in accordance with guidelines and regulations approved by Wenzhou University.

## STATISTICAL ANALYSIS

Data was analyzed with software SPSS 16.0. The data are shown as the mean ± SEM of three independent trials. Statistical comparisons were made by one-way ANOVA followed by Tukey's test. Statistical significance was set at $P<0.05$, $P<0.01$ or $P<0.001$.

## RESULTS

### *Geniposide reduces $H_2O_2$-induced apoptosis in PC12 cells*

We determined the effects of different concentrations of geniposide on the survival rate of PC12 cells by using the MTT assay. Geniposide was incubated with cells at the following concentrations: 0mg/l, 10mg/l, 20mg/l, 50mg/l, and 100mg/l. The results showed that when the concentration of geniposide was greater than 50mg/l, the survival rate of PC12 cells reached more than 50%. However, when the geniposide concentration was less than 50mg/l, the cell survival rate was less than 50%. This result indicates that the neuroprotective effects of geniposide against apoptosis of PC12 cells in the presence of $H_2O_2$ (1mM) is concentration-dependent (fig. 1).

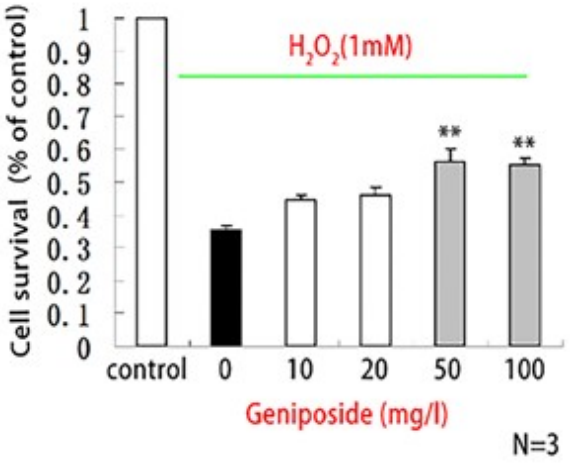


**Fig. 1**: PC12 cells were pretreated with geniposide and exposed to 1mM $H_2O_2$. Cell viability was measured by the MTT assay. The values are expressed as the mean ± SEM. Percentage of vehicle treatment (N=3). **p<0.01 versus vehicle treatment.

***Exogenously applied geniposide affects iLTP***

To investigate whether and how exogenously applied geniposide affects iLTP, different concentrations of geniposide (L-Geni (200mg/l), M-Geni (500mg/l) and H-Geni (1000mg/l)) (fig. 2) together with OGD were administered to hippocampal slices for 10min. Both L-Geni (n=6, p<0.001; fig. 2) and M-Geni (n=10, p<0.001; fig. 2) significantly abolished iLTP by decreasing the fEPSP slope. However, compared with the OGD group the (n=6, fig. 2), the H-Geni group (n=5, fig. 2) failed to exhibit decreased iLTP. These results are the first to reveal that treatment with geniposide can attenuate postischemic LTP induction in a concentration-dependent manner.

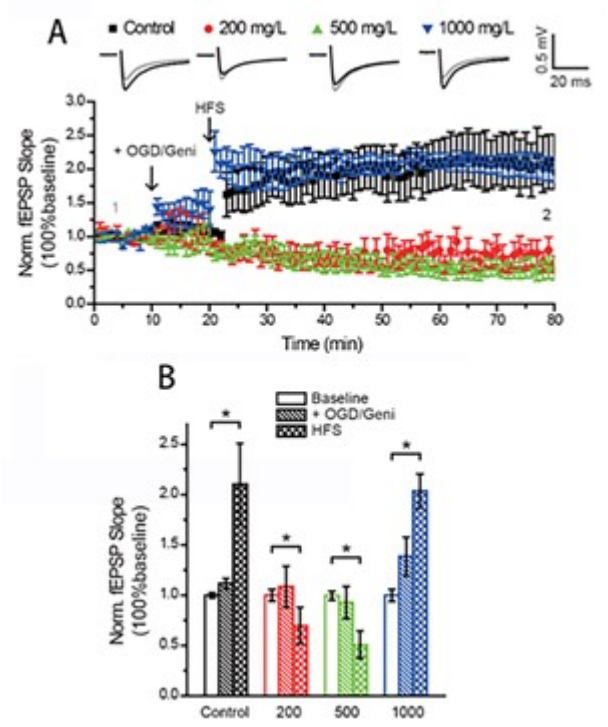


**Fig. 2**: Dose-dependent suppression of iLTP by geniposide during OGD-induced ischemia. A: Representative fEPSP slopes following iLTP induced by OGD, 200 mg/l (L-Geni; normalized slope 1.40347±0.35366, n=6, p<0.001), 500 mg/l (M-Geni; normalized slope 1.59375±0.31632, n=10, p<0.001) and 1000 mg/l (H-Geni; normalized slope 0.06613, n=5). B: Summary of the data showing the effects on iLTP in each group. The bar represents the mean ± SEM of the samples. * P<0.001 vs baseline.

***Neuroprotective effect of geniposide is regulated by the GluN2A receptor in hippocampal slice cultures***

The NMDA receptor is responsible for stroke-triggered cell death. GluN2A and GluN2B are the major constituents of NMDA receptors. To investigate the pathway underlying the effects of geniposide *in vitro*, we recorded fEPSP evoked by different antagonists in hippocampal CA1 neurons under OGD conditions. After 10 min of baseline recording, 10 min of OGD/geniposide perfusion persistently decreased the fEPSP slope over 60 min (fig. 3). This electrophysiological finding is consistent with previous studies on iLTP in ischemia (Chen *et al.,* 2015). While geniposide with co-administered with the NR2A inhibitor NVP (n=6, p<0.001, fig. 3), iLTP was significantly elevated due to an increase in the fEPSP slope. However, compared with the administration of geniposide alone (n=6, fig. 3.), the administration of geniposide with the NR2B blocker ifenprodil (n=6, fig. 3.) failed to invert iLTP. These results suggest that treatment with geniposide can attenuate postischemic LTP induction through NR2A-containing NMDARs.

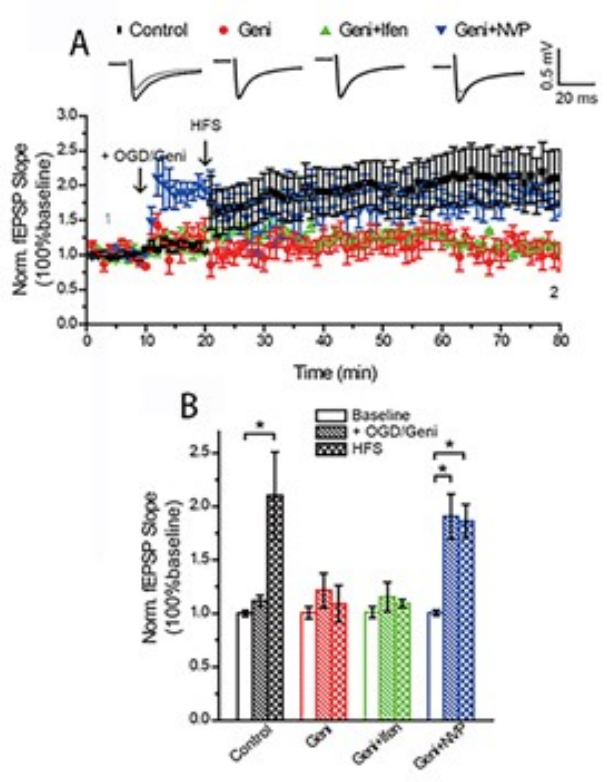


**Fig. 3**: Effects of geniposide on ischemia in vitro. A: Representative fEPSP slopes following iLTP induced by OGD used to study the effect of geniposide on iLTP after ischemia. The samples were divided into the geniposide, geniposide + ifenprodil (Ifen), and geniposide + N-vinylpyrrodidone (NVP) groups. OGD/geniposide perfusion was performed 10 min after baseline recording, high-frequency stimulation (HFS) was performed for 20 min, and fEPSP slopes were recorded. B：Summary of the data showing the effects of iLTP in each group. The bar represents the mean ± SEM of the samples. * P<0.001 is showing comarison against baseline.

## DISCUSSION

*Gardenia jasminoides Ellis*, a plant of the Rubiaceae family, is a famous iridoid glycoside. Among iridoid glycoside compounds, geniposide has been found to be

associated with heat clearance and detoxification (Li *et al.,* 2013). Previous studies have demonstrated that geniposide has potential protective effects against neurodegenerative disorders (Li-xia *et al.,* 2009), especially cellular death after stroke (Lee *et al.,* 2006, Yamazaki *et al.,* 2001). However, whether and how geniposide inhibits neuronal death through NMDAR-dependent antioxidative effects remain unclear. Here, we report that geniposide may be a potent treatment for the amelioration of ischemic neuronal injury *in vitro*.

In the current study, we found that geniposide at medium and high doses increased PC12 cell viability during oxidative stress, which indicated the protective effect of geniposide against I/R-induced cerebral injury in vitro. In addition, we showed that 200mg/l and 500mg/l geniposide greatly decreased iLTP in hippocampal CA1 neurons subjected to OGD. We demonstrated that the neuroprotective effect of geniposide is concentration-dependent and these results are consistent with our previous findings in ischemia *in vivo*.

To date, it has not been fully elucidated whether and how geniposides are involved in neuroprotection via NMDAR-dependent antioxidative mechanisms. Therefore, we investigated the protective mechanism of geniposides against hippocampal CA1 neurons under OGD conditions. In particular, we explored the function of NMDARs and their subtypes by applying different antagonists of NMDAR subunits to geniposide-treated neurons.

During pathological conditions such as stroke, the excessive stimulation of NMDARs and subsequent neuronal nitric oxide synthase (nNOS) activation are highly important for triggering neuronal injury (Lee *et al.,* 1999, Lipton 2007). In NMDAR-nNOS pathways, the modulation of the ratio of GluN2A/GluN2B-containing NMDARs is believed to be a crucial element of neuronal survival and death (Sun *et al.,* 2017). In our present work, we observed that treating OGD-exposed hippocampal slices with geniposide attenuated the death of hippocampal CA1 neurons. However, which NMDAR subtypes contribute to this protective effect is unknown. Indeed, we proves that cotreatment with geniposide and the GluN2A antagonist NVP reversed the protective effect of geniposide alone. In contrast, the protective effect of geniposide was not influenced by cotreatment with the GluN2B inhibitor ifenprodil. Therefore, we suggest that GluN2A contributes to the protective role of geniposide(Sun *et al.,* 2018). Many studies have reported that NMDARs are potential therapeutic targets for reducing brain damage in rodent models of ischemic stroke(Liu *et al.,* 2007, Takahashi *et al.,* 2015). To date, clinical trials of NMDAR inhibitors in human stroke patients have not yet succeeded. This is probably due to the series of acute side effects induced by the direct suppression of NMDARs, which play significant physiological roles in the CNS. Dong-Ya Zhu *et al.,*. confirmed that NMDAR-2B (NR2B)-dependent signaling induces PSD-95, which is a scaffolding protein that binds both NMDARs and nNOS at excitatory synapses. They developed a small-molecule inhibitor to block the NMDAR-PSD95-nNOS pathway (Zhou *et al.,* 2010). Importantly, this drug was proven to be a very effective agent with both robust neuroprotective activity in vitro and the ability to improve focal cerebral damage induced by middle cerebral artery occlusion and reperfusion in rodents without having major side effects (Jones 2011). However, other reports have argued that its antioxidative effects are due not only to the NR2B-dependent death-releasing pathway but also to NR2A-dependent survival-triggering signaling (Chen *et al.,* 2015, Y Liu *et al.,* 2007, Yao *et al.,* 2012). Based on our results, geniposide has high potential as a bioactive compound against oxidative stress via NR2A-containing NMDARs in ischemia. Indeed, a pilot study showed that geniposide may have antioxidative effects through PI3K/Akt signaling in hippocampal neurons (Huang *et al.,* 2017, Jiang *et al.,* 2016, Yin *et al.,* 2010).

Vast numbers of studies have revealed that potential therapeutic agents reduce post-ischaemic damage through deactivation of NMDARs in animal stroke models (Lai *et al.,* 2014, Lipton (2007). Until now, however, it has been proved that those compounds are failed in the application of human stroke subjects (Albers *et al.,* 2001, Davis *et al.,* 1997). There are many reasons they have failed, but the largest one is that chemically synthesized compounds are inevitable with side effects. NMDAR dysfunctions and LTP impairments are the common strategies during the early stage of post-stroke injuries, nevertheless, loss or partial block of NMDAR will affect the basic motor excitability and ultimately lead to insufficient potentiation in the rehabilitation (Brown *et al.,* 2020). According to our findings, geniposide treatment rescued the i-LTP impairment after stroke and acted on the elevation of NR2A-dependent pathways, suggesting that the protective effect of geniposide is selectively alleviate excitotoxicity without affecting the basal synaptic transmission.

Therefore, it is quite reasonable that geniposide may enhance antioxidant capacity by elevating the NR2A pathway and subsequently activating downstream PI3K/Akt factors in ischemia. Nevertheless, we cannot exclude the possibility that geniposide can alleviate other death-related pathways based on our data on NMDARs. More research should focus on the efficiency of geniposide as a prominent antioxidant through NMDARs.

## CONCLUSION

In conclusion, we demonstrated that geniposide is neuroprotective against ischemia in a concentration-dependent manner.

We also found that geniposide ameliorates iLTP by enhancing GluN2A-containing NMDARs. Our data indicate that geniposide is a highly promising therapeutic compound for protecting against neurodegenerative diseases, such as stroke.

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