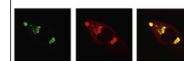


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Research Report

Neuroprotection by genipin against reactive oxygen and reactive nitrogen species-mediated injury in organotypic hippocampal slice cultures



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ABSTRACT

Genipin, the multipotent ingredient in *Gardenia jasmenoides* fruit extract (GFE), may be an effective candidate for treatment following stroke or traumatic brain injury (TBI). Secondary injury includes damage mediated by reactive oxygen species (ROS) and reactive nitrogen species (RNS), which can alter the biological function of key cellular structures and eventually lead to cell death. In this work, we studied the neuroprotective potential of genipin against damage stemming from ROS and RNS production in organotypic hippocampal slice cultures (OHSC), as well as its potential as a direct free radical scavenger. A 50 μ M dose of genipin provided significant protection against tert-butyl hydroperoxide (tBHP), a damaging organic peroxide. This dosage of genipin significantly reduced cell death at 48 h compared to vehicle control (0.1% DMSO) when administered 0, 1, 6, and 24 h after addition of tBHP. Similarly, genipin significantly reduced cell death at 48 h when administered 0, 1, 2, and 6 h after addition of rotenone, which generates reactive oxygen species via a more physiologically relevant mechanism. Furthermore, genipin significantly reduced both cell death and nitrite levels at 24 h caused by S-nitroso-N-acetylpenicillamine (SNAP), a direct nitric oxide (NO) donor, and successfully quenched 1,1-Diphenyl-2-picryl-hydrazyl (DPPH), a stable free radical, suggesting that genipin may act as a direct free radical scavenger. Our encouraging findings suggest that genipin should be tested in animal models of CNS injury with a significant component of ROS- and RNS-mediated damage, such as TBI and stroke, to assess its *in vivo* efficacy.

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1. Introduction

Gardenia jasmenoides fruit extract (GFE) has been used in traditional Chinese medicine as a treatment for various

maladies. It is now commonly believed that genipin is the multipotent ingredient in GFE. Genipin is a hydrolyzed metabolite of geniposide, due to the action of β -D-glycosidases, which are present in the intestines and liver

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(Akao et al., 1994). Genipin has been reported to protect against amyloid β toxicity (Yamazaki et al., 2001), reduce inflammation in a rat paw edema model (Koo et al., 2006), attenuate diabetic neuropathy (Qiu et al., 2012), and increase neurite outgrowth in Neuro2a cells (Yamazaki and Chiba, 2008), which collectively suggest that genipin may have promise as a candidate for treatment following traumatic brain injury (TBI) or stroke. Although genipin is well-known for its cross-linking abilities and as a coloring agent (Jin et al., 2004; Yuan et al., 2007), its neuroprotective effects following brain injury have not been tested in complex *in vitro* models.

Secondary processes initiated by brain injury set in motion an extended cascade of pathological consequences, including damage by reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Smith et al., 1994). After brain injury, superoxide radical concentration increases significantly, due to many factors, including enzyme disruption (Bayir et al., 2006), mitochondrial dysfunction (Zhang et al., 2006), and lipid peroxidation (Koo et al., 2004). Superoxide can then react with nitric oxide (NO) to create the particularly reactive free radical peroxynitrite. The NO concentration increases following brain injury, due to the activity of nitric oxide synthases (NOS), and is thus readily available for this reaction (Cherian et al., 2004). Superoxide can also react to form hydrogen peroxide, which can then undergo a radical reaction to produce the hydroxyl radical (OH \cdot), which is also particularly damaging (Bayir et al., 2006). Brain injury often involves hemorrhage, which elevates levels of heme-iron groups and other transition metals in the parenchyma, which then catalyze additional radical-generating reactions and further increase ROS and RNS (Lewen et al., 2000; Wagner et al., 2003). ROS and RNS can cause damage to DNA/RNA, lipids, proteins, and the blood brain barrier, and eventually lead to cell death (Smith et al., 1994).

To study the potential of genipin treatment in the setting of brain injury, we have focused on whether genipin was protective following ROS- and RNS-mediated damage to organotypic hippocampal slice cultures (OHSC). ROS-mediated damage was produced using tert-butyl hydroperoxide (tBHP). Genipin's effect against nitric oxide toxicity was explored using the direct NO donor S-nitroso-N-acetylpenicillamine (SNAP). Rotenone was also used to generate reactive oxygen species from within the tissue by inhibiting Complex I of the electron transport chain. Additionally, genipin was tested for direct free radical scavenging ability.

2. Results

2.1. Genipin toxicity curve

Exposing OHSC to several doses of genipin revealed that 500 μ M genipin was toxic, while 50 μ M and 5 μ M caused minimal cell death. Vehicle treated cultures received 1% DMSO, the maximum DMSO concentration for the group treated with 500 μ M genipin in this experiment only (Fig. 1).

2.2. Genipin treatment following tBHP injury

Of the doses tested, the greatest protection against tBHP injury was provided by 50 μ M genipin, which significantly

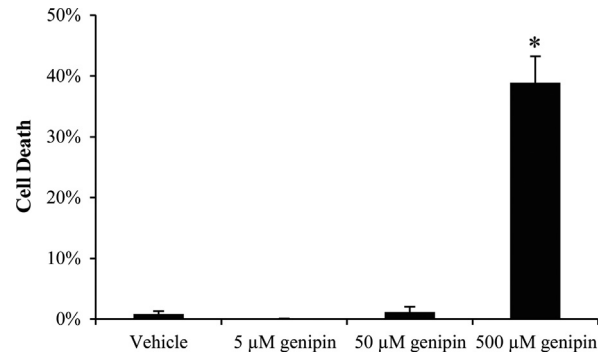


Fig. 1 – Genipin toxicity curve. Cell death was measured 48 h after addition of genipin at the indicated concentrations. Genipin was replenished at 24 h (* $p < 0.05$ compared to vehicle, $n \geq 12$ per group).

reduced cell death compared to vehicle control (0.1% DMSO) (Fig. 2A). Treatment with 50 μ M genipin was more protective than 3 mM ascorbic acid. In fact, treatment with 50 μ M ascorbic acid significantly enhanced the cytotoxicity of tBHP at 48 h compared to vehicle treated cultures. Lower doses of 5 μ M and 0.5 μ M also significantly reduced cell death, albeit to a lesser extent (Fig. 2A). Treatment with 50 μ M genipin significantly reduced cell death when treatment was delayed 1 h, 6 h, or 24 h after tBHP injury onset, compared to vehicle treatment (0.1% DMSO), whereas 50 μ M genipin treatment administered at 36 h did not provide significant protection (Fig. 3A).

2.3. Genipin treatment following rotenone injury

Treatment with 50 μ M genipin significantly reduced cell death following injury by the Complex I inhibitor rotenone compared to vehicle (0.1% DMSO). Genipin significantly reduced cell death when treatment was delayed 0, 1, 2 and 6 h after injury, but was no longer effective when treatment was delayed 24 h (Fig. 3B).

2.4. Genipin treatment following SNAP injury

Treatment with 50 μ M genipin significantly reduced both cell death and nitrite levels produced by 10 mM SNAP compared to vehicle treatment (0.1% DMSO, Fig. 4A). Lower concentrations of SNAP did not produce cell death (data not shown). However, in response to lower concentrations of SNAP, 50 μ M genipin significantly reduced nitrite concentrations after treatment with either 250 μ M or 500 μ M SNAP. Genipin did not significantly reduce nitrite concentrations after a 1 mM SNAP challenge (Fig. 4B).

2.5. DPPH assay

Treatment with 50 μ M genipin successfully quenched 50 μ M DPPH radical, demonstrating direct free radical scavenging abilities similar to the well-known antioxidant ascorbic acid (50 μ M) (Fig. 5).

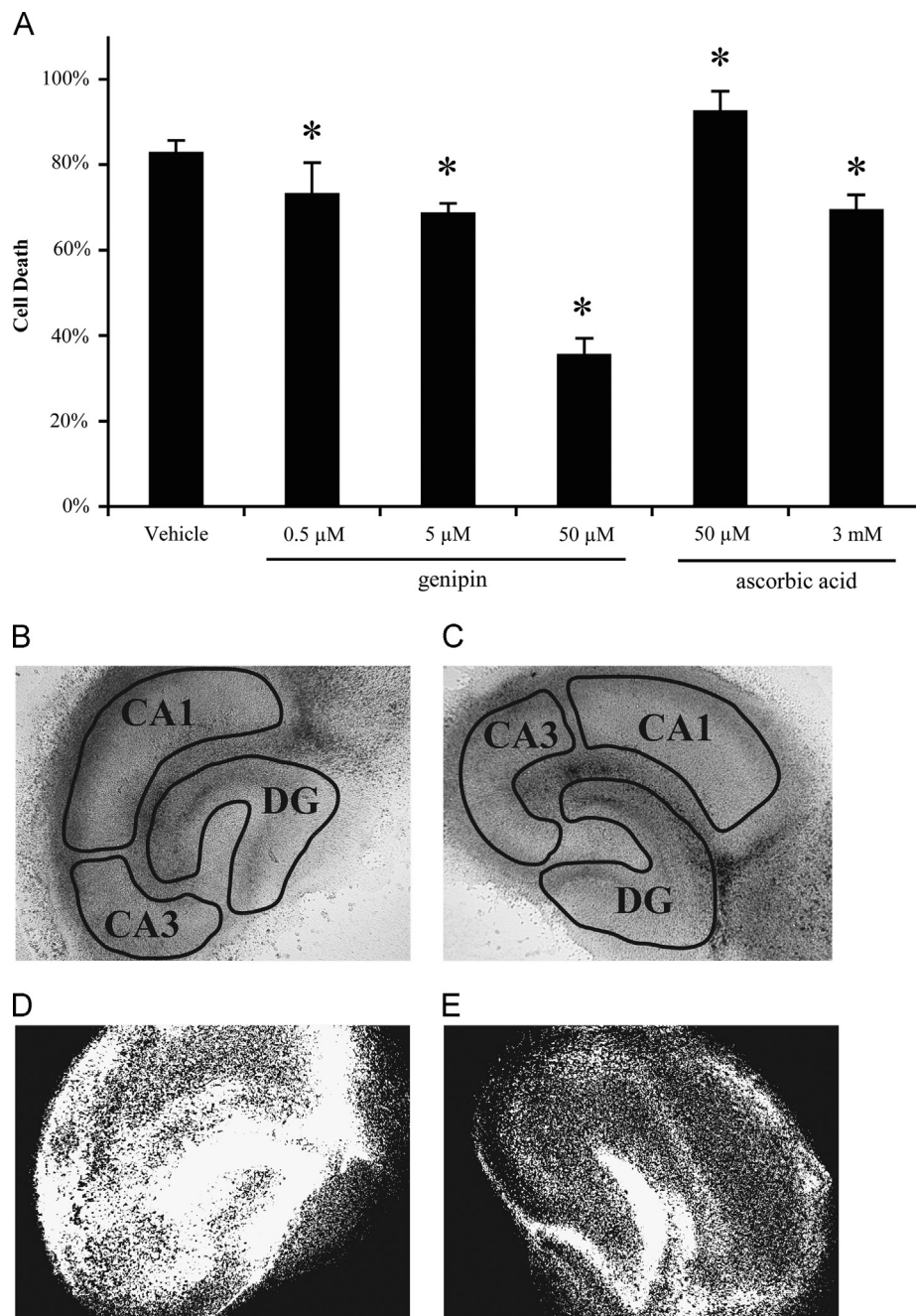


Fig. 2 – (A) Genipin dose response at 48 h after injury onset. Doses of 0.5 μM, 5 μM, and 50 μM genipin were added at onset of 24 h 1 mM tBHP injury and replenished at 24 h. Doses of 50 μM and 3 mM ascorbic acid were used as a positive control. Cell death was measured 48 h after injury onset ($*p < 0.05$ compared to vehicle, $n \geq 8$ per group). **(B)** Bright field image of OHSC before 1 mM tBHP injury and vehicle treatment, with regions outlined. **(C)** Bright field image of OHSC before 1 mM tBHP injury and 50 μM genipin treatment, with regions outlined. **(D)** Thresholded Sytox Green stained OHSC 48 h after 1 mM tBHP injury and vehicle treatment, showing cell death. **(E)** Thresholded Sytox Green stained OHSC 48 h after 1 mM tBHP injury and 50 μM genipin treatment, showing reduced cell death.

3. Discussion

In the present study, genipin conferred significant protection against multiple forms of ROS and RNS stress imposed on OHSCs. Because of the prevalence of ROS- and RNS-mediated injury mechanisms in multiple forms of brain injury, our results suggest that the neuroprotective potential

of genipin should be explored in animal models of TBI and stroke.

Genipin was more neuroprotective against tBHP than either high or low concentrations of the common antioxidant ascorbic acid. Genipin may be advantageous over ascorbic acid not only because it provided greater protection against tBHP, but also because it lacked the toxic pro-oxidant characteristics

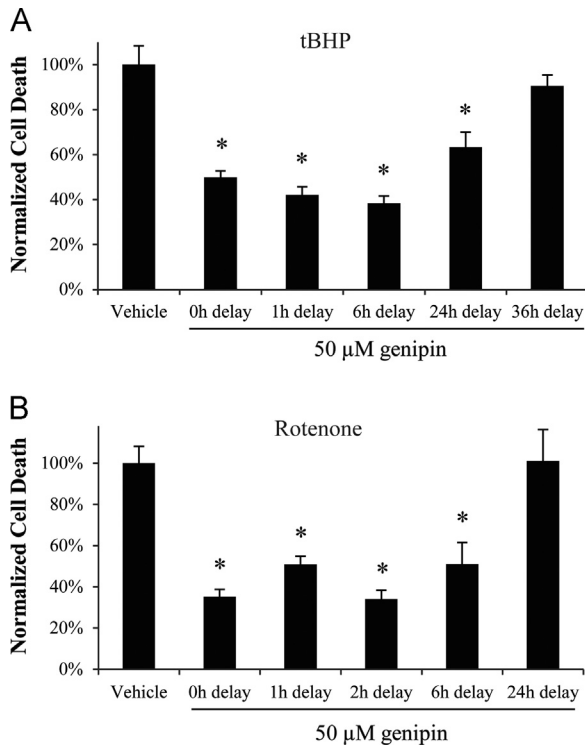


Fig. 3 – Genipin delayed treatment. (A) Normalized percent cell death following 24 h 1 mM tBHP injury. 50 μM genipin treatment was delayed 0, 1, 6, 24, and 36 h after injury onset. Cell death was measured at 48 h after injury onset (**p* < 0.05 compared to vehicle, *n* ≥ 12 per group). **(B)** Normalized percent cell death following 1 h 10 μM rotenone injury. 50 μM genipin treatment was delayed 0, 1, 2, 6, and 24 h after injury onset (**p* < 0.05 compared to vehicle, *n* ≥ 8 per group).

of ascorbic acid at lower concentrations. Although a higher concentration of 3 mM ascorbic acid was protective against tBHP, such high concentrations are not therapeutically practical, with extracellular and plasma concentrations of ascorbic acid plateauing at 70–80 μM upon increasing dietary intake (Padayatty et al., 2003). Several common antioxidants, including ascorbic acid become pro-oxidants at lower concentrations, especially in the presence of iron *in vivo* (Yen et al., 2002). It has also been shown that antioxidants such as isothiocyanates (Valgimigli and Iori, 2009), various tea extracts (Yen et al., 1997), and numerous water-soluble food-related botanical extracts (Damien Dorman and Hiltunen, 2011) exhibit similar pro-oxidant properties at low concentrations, even in the absence of iron. Thus, compared to common antioxidants, genipin may be a stronger, safer, and more consistent alternative for protection against oxidative damage following brain injury. Indeed, few antioxidants have been found effective in clinical trials for treatment against TBI (Faden, 2001; Rigg et al., 2005).

In addition to protecting against ROS and RNS generated in the surrounding medium, genipin protected against oxidative stress originating within the OHSC, specifically within the mitochondria. Disruption of the mitochondrial electron transport chain by rotenone results in the direct formation of

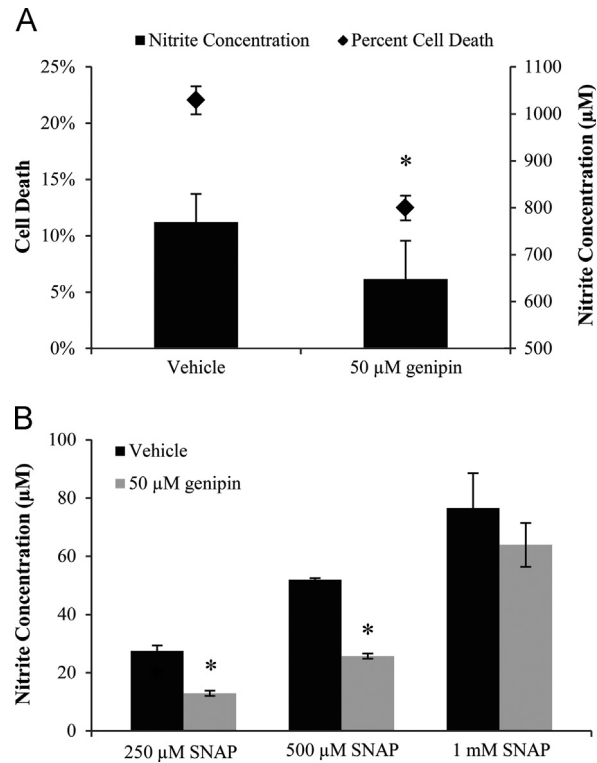


Fig. 4 – Cell Death and nitrite concentrations following SNAP injury. (A) Cell death (**p* < 0.05 compared to vehicle, *n* ≥ 8 per group) and nitrite concentration (**p* < 0.05 compared to vehicle, *n* ≥ 6 per group) were measured 24 h after addition of 10 mM SNAP. **(B)** Nitrite concentration measured 24 h after addition of 250 μM, 500 μM, or 1 mM SNAP with or without genipin (**p* < 0.05 compared to vehicle, *n* ≥ 2 per group).

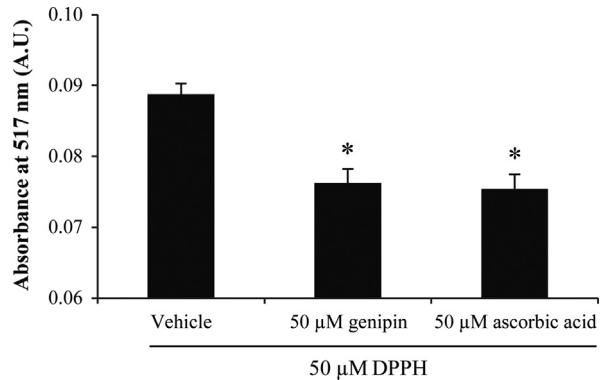


Fig. 5 – DPPH assay. Doses of 50 μM genipin or 50 μM ascorbic were added to 50 μM DPPH free radical. Absorbance was measured 30 minutes after addition (p* < 0.05 compared to vehicle, *n* ≥ 12 per group).**

superoxide radicals within the mitochondria. Genipin significantly reduced cell death due to rotenone injury, providing promise for genipin's ability to permeate within cells to prevent the widespread damage following the internal production of ROS and RNS. Thus, rather than merely preventing damage caused by ROS and RNS in the surrounding medium, genipin protected against a similar source of oxidative stress

to that seen in brain injury, specifically, functionally and structurally compromised mitochondria (Lifshitz et al., 2003).

Often patients who experience a TBI or stroke are unable to receive immediate treatment and may not reach medical assistance for hours after injury. Therefore, enthusiasm for experimental translation is diminished if compounds do not exhibit significant neuroprotection in a delayed treatment paradigm. In the current study, genipin significantly reduced cell death caused by tBHP and rotenone when administered up to 6 h after injury onset. Although the temporal correlation between oxidative damage *in vitro* and *in vivo* has not been assessed in detail, following cortical impact injury in the mouse, significant oxidative damage to mitochondria occurs within 3–6 h post-injury (Singh et al., 2006). Taken together, these results suggest that genipin might be protective *in vivo* as well, even when treatment is delayed several hours. At the very least, our results suggest that follow up in an *in vivo* model of brain injury with a substantial oxidative component is warranted.

Although genipin decreased nitrite levels and cell death following SNAP injury, its mechanism of action regarding NO toxicity remains elusive. Contrary to our findings, genipin has been reported to increase nitric oxide levels by activating the NO-cGMP-PKG signaling pathway, followed by ERK phosphorylation in both Neuro2a and PC12h cells (Yamazaki and Chiba, 2008). Genipin is hypothesized to increase NO in this pathway by directly binding and activating neuronal nitric oxide synthases (nNOS) (Ohkubu et al., 2004). In that study, increased nNOS activity, and a subsequent increase in NO, resulted in neurite outgrowth. Additionally, other findings suggest genipin decreased expression of inducible nitric oxide synthases (iNOS), which contribute to the toxic spike in NO levels following brain injury. Genipin decreased iNOS expression by both interfering with the NF- κ B/I κ B- β pathway (Koo et al., 2004) and by up-regulating heme oxygenase-1 (HO-1) (Jeon et al., 2011). Thus, our results, which indicate that genipin lowered nitrite levels, are consistent with previous results reporting down-regulation and inhibitory effects on iNOS, but not with contradictory results reporting up-regulation of nNOS. However, in an injurious situation, genipin's net effect on iNOS and nNOS may result in a total decrease in NO production, a decrease in the surrogate marker nitrite, and hence improved outcome after injury.

As suggested by the DPPH results of this study, genipin may also reduce ROS and RNS by directly quenching free radicals. At 50 μ M, genipin demonstrated an ability to scavenge free radicals comparable to ascorbic acid. Our results are in contrast with previous studies that have shown genipin exhibits little (Lee et al., 2009) or almost no free radical scavenging ability in the DPPH assay (Koo et al., 2006). When compared to gardenia fruit extract (GFE), genipin had little free radical scavenging ability, with an $IC_{50} > 150 \mu$ g/mL, versus an $IC_{50} = 38 \mu$ g/mL for GFE (Lee et al., 2009). Differences in experimental setup, genipin concentration, genipin source, and solvent could explain the discrepancy with our study. Our results lead to new conclusions about one potential mechanism of neuroprotection by genipin. As a direct free radical scavenger, genipin has the potential to provide immediate and widespread protection following brain injury, without the deleterious effect of enhancing free radical

damage at low concentrations in physiological conditions as ascorbic acid did in the present study.

In addition to direct free-radical scavenging, genipin may decrease the effects of brain injury *in vivo* through other mechanisms. Genipin significantly reduced the lipid peroxidation product malondialdehyde (Koo et al., 2004). Furthermore, genipin increased the expression of endogenous heme oxygenase-1 (HO-1) in murine macrophage cell lines (Jeon et al., 2011), thus preventing oxidative damage. Although genipin possesses many beneficial effects, translation *in vivo* may be hampered by its anti-coagulant effects. In the mouse genipin administration significantly prolonged the time required for thrombotic occlusion and inhibited collagen-induced platelet aggregation (Suzuki et al., 2001). Genipin's anti-thrombotic effects have further been shown to inhibit exocytosis *in vivo* and prolong bleeding time (Wang et al., 2009). Given these potential negative side effects, future *in vivo* studies with genipin should monitor for coagulopathies. It is also unclear if genipin is able to cross the blood brain barrier (BBB). Geniposide, the precursor to genipin, does not readily cross the healthy BBB. However genipin has significantly different drug metabolism from geniposide (Hou et al., 2008; Tseng and Tsai, 2004). After many types of CNS injuries including TBI and stroke, the BBB is compromised, and compounds in the serum can enter the parenchyma (Neuwelt et al., 2008). Under these conditions, a compound as small as genipin (226 Da) can easily penetrate into the brain tissue where it is needed. The ability of genipin to cross the intact BBB remains to be studied in detail *in vivo*.

In conclusion, genipin shows promise as a treatment following *in vitro* brain injury. With a therapeutic dose 10 times smaller than a damaging dose, its therapeutic index is encouraging for *in vivo* safety. Genipin has a long therapeutic window, conferring protection up to 6 h after tBHP and rotenone injury onset. Genipin not only significantly reduced cell death due to ROS- and RNS-mediated injury, but reduced total nitrite concentration following SNAP treatment. Therefore, it may have benefit in multiple disease- or injury-states with a significant component of ROS- and RNS-mediated damage, including TBI, ischemia, or hemorrhage. It would be advantageous to study genipin in greater detail and in more complex models to confirm its protection in OHSCs and to clarify its overall effect in various models of brain injury.

4. Experimental procedures

4.1. Organotypic hippocampal slice cultures

All animal procedures were approved by the Columbia University IACUC. Organotypic hippocampal slice cultures (OHSC) were prepared from post-natal day 9–10 Sprague-Dawley rat pups as described previously (Morrison et al., 2002; Stoppini et al., 1991). Hippocampi were removed aseptically, cut into 400 μ m slices using a McIlwain Tissue Chopper (Mickle Laboratory Engineering, Surrey, UK), and plated on Millicell-CM tissue culture inserts (4 slices per insert, Millipore, Billerica, MA). Slices were cultured in Neurobasal medium (Life Technologies, Grand Island, NY), supplemented with B-27 (Life Technologies), 5 mg/mL D-glucose, and 1 mM/L

glutamax (Sigma-Aldrich, St. Louis, MO) for two days before being switched to a full serum medium (FSM) consisting of 50% minimum essential medium (MEM), 25% heat-inactivated horse serum, and 25% Hank's balanced salt solution (Morrison et al., 2002). Medium was replenished every 2–3 days by replacing half with fresh FSM. Cultures were maintained at 100% humidity, 5% CO₂, 37 °C and used for experiments after 11 days *in vitro*. At the time of experimentation, FSM was replaced with serum free medium consisting of 75% MEM, 25% Hank's balanced salt solution, and B-27 supplement and treated with 75 µg/mL gentamicin (Life Technologies) to reduce risk of infection. At the indicated times, cultures were stained with 5 µM Sytox Green (Life Technologies) in SFM for 30 min and then imaged with an excitation filter of 492 ± 9 nm and an emissions filter of 530 ± 10 nm (Chroma Technology, Bellows Falls, VT) to quantify cell death with MetaMorph image analysis software (Molecular Devices, Downingtown, PA). Cell death was calculated as the percent area staining above a given threshold in a given anatomical region, dentate gyrus (DG), CA3, CA1, as previously described (Morrison et al., 2002). Any OHSC with greater than 1% cell death pre-injury were eliminated from the study as being unhealthy.

4.2. Genipin toxicity curve

OHSC were treated with 0.5 µM, 5 µM, 50 µM, or 500 µM genipin in DMSO (Sigma) and stained with Sytox Green at 24 and 48 h to determine the toxicity profile of genipin in naïve cultures.

4.3. Tert-butyl hydroperoxide injury

Tert-butyl hydroperoxide (tBHP) (Sigma) was applied to OHSC at a dose of 1 mM for 24 h to induce direct ROS-mediated injury. This radical producer is an organic peroxide that is favored over hydrogen peroxide in models of oxidative stress due to its ability to induce consistent and sustained oxidative cellular stress (Alia et al., 2005). OHSC hippocampal slices were simultaneously treated with the indicated compound or the appropriate vehicle. At 24 h, the tBHP was removed and OHSC were treated with fresh drug or vehicle. OHSC were imaged pre-injury with Sytox Green and again at 48 h. The three safe doses of genipin (0.5 µM, 5 µM, and 50 µM) were tested in this tBHP injury model to determine which concentration resulted in the greatest protection. The most effective concentration tested (50 µM) was then used in a delayed treatment paradigm to determine the therapeutic window of genipin protection after tBHP injury. Treatment was delayed for 0, 1, 6, 24, or 36 h after tBHP injury onset, and cell death was measured at 48 h.

4.4. Rotenone

Rotenone (Sigma) inhibits complex I of the mitochondrial electron transport chain by blocking the transfer of electrons, thereby producing ROS and RNS in a more physiologically relevant form than tBHP. A 10 µM dose of rotenone was applied to OHSC for 1 h and then removed (Guangpinx et al., 2003). OHSC were treated simultaneously with 50 µM

genipin or vehicle (0.1% DMSO) and imaged pre-injury with Sytox Green and at 48 h. A delayed treatment paradigm was implemented to determine the therapeutic window of genipin protection after rotenone injury. Treatment was delayed for 0, 1, 2, 6, or 24 h after rotenone injury onset.

4.5. SNAP Injury

S-Nitroso-N-acetylpenicillamine (SNAP) (Sigma) is a direct nitric oxide (NO) donor. NO-mediated stress was induced by incubating OHSC in 10 mM SNAP for 24 h, and OHSC were stained with Sytox Green pre-injury and at 24 h post-injury. Lower concentrations of SNAP (250 µM, 500 µM, and 1 mM) did not induce cell death, but did increase nitrite concentration. The amount of nitrite present after SNAP injury was determined using the Griess method (Guevara et al., 1998). NO is rapidly oxidized by oxygen in living systems to form nitrite. Nitrite levels can be measured to assess the amount of NO being produced, due to its relative stability compared to NO (Granger et al., 1996). Thus, nitrite levels measured by the Griess assay provide insight into the toxic NO levels in the tissue, which are in equilibrium with the particularly potent radical peroxynitrite. According to the manufacturer's instructions, 150 µL media from each well was mixed with 20 µL Griess reagent (Life Technologies) in 130 µL distilled water. After 30 min, absorbance was measured at 548 nm on a Synergy4 Multi-mode Microplate reader (BioTek Instruments, Winooski, VT) and converted to nitrite concentrations with a standard curve.

4.6. DPPH assay

1,1-Diphenyl-2-picryl-hydrazyl (DPPH) (Sigma) was used to measure the free radical scavenging effects of 50 µM genipin. DPPH is a stable free radical that is a deep violet color but becomes clear when reduced (Sharma and Bhat, 2009). Absorbance was read at 517 nm on a Synergy4 Multi-mode Microplate reader (BioTek Instruments) 30 min after adding 50 µM genipin, 50 µM ascorbic acid, or vehicle to 50 µM DPPH.

4.7. Statistical analysis

Data are presented as the mean ± SEM. Significance was determined by analysis of variance (ANOVA) followed by post-hoc tests, either Bonferroni's or Dunnett's tests as appropriate using the Data Analysis Add-On, Excel (Microsoft, Redmond, WA). Significance was set at $p < 0.05$.

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