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Champagne Wine Polyphenols Protect Primary Cortical Neurons against Peroxynitrite-Induced Injury

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White wines are generally low in polyphenol content as compared to red wines. However, Champagne wines have been shown to contain relatively high amounts of phenolic acids that may exert protective cellular actions in vivo. In this study, we have investigated the potential neuroprotective effects of Champagne wine extracts, and individual phenolics present in these extracts, against peroxynitrite-induced injury. Organic and aqueous Champagne wine extracts exhibited potent neuroprotective activity against peroxynitrite-induced injury at low concentrations (0.1 μg/mL). This protection appeared to be in part due to the cellular actions of individual components found in the organic extracts, notably tyrosol, caffeic acid, and gallic acid. These phenolics were observed to exert potent neuroprotection at concentrations between 0.1 and 10 μM. Together, these data suggest that polyphenols present in Champagne wine may induce a neuroprotective effect against oxidative neuronal injury.

KEYWORDS: Cortical neurons; protective effect; Champagne wine; peroxynitrite; phenolics

INTRODUCTION

Oxidative insults to neuronal cells have been implicated in the pathogenesis of neurodegenerative disorders such as Alzheimer's and Parkinson's diseases (*1*, *2*). The generation of reactive oxygen species, such as superoxide ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2), in mitochondria or via the autoxidation of catecholamines has been proposed to contribute to neuronal injury (*3*). Furthermore, the colocalization of nitrogen monoxide (NO) and superoxide ($O_2^{\bullet -}$) within neurons may lead to the formation of peroxynitrite ($ONOO^-$) (*4*), which may induce the oxidation of DNA, lipids, and protein sulfhydryls or the nitration of DNA and phenolic compounds such as tyrosine (*5*). Indeed, levels of 3-nitrotyrosine have been shown to be elevated in a variety of neurodegenerative diseases (*6*) and has been found in brain tissue from Parkinson's disease patients (*7*), suggesting that peroxynitrite and other reactive nitrogen species may play a role in the pathophysiology of these diseases.

There has been much recent interest in the potential of plant-derived polyphenols to protect against neuronal injury. Regular moderate consumption of red wine has been shown to be beneficial by counteracting cerebral aging (*8*) and by inducing cardioprotective effects (*9*). Flavonoids have been observed to protect against both age-related cognitive and motor decline (*10*) and against 6-hydroxydopamine neurotoxicity and MPTP lesioning of the nigrostriatal tract (*11*). Although polyphenols such as flavonoids are powerful hydrogen-donating antioxidants and scavengers of reactive nitrogen species in vitro (*12*), recent findings demonstrate a role for specific flavonoids in interacting selectively within signaling cascades that regulate neuronal survival following exposure to oxidative stress (*13*). For example, accumulating evidence suggests that flavonoids might exert neuroprotective effects at nanomolar concentrations through the selective modulation of both protein kinase and lipid kinase signaling cascades, such as tyrosine kinase, PI 3-kinase/Akt, PKC, and mitogen-activated protein (MAP) kinase pathways (*14*). This, along with evidence that dietary polyphenols can cross the blood brain barrier (*15*), suggests that such dietary components have the potential to act within the central nervous system.

Champagne wine has been shown to contain relatively high amounts of phenolics such as tyrosol and caffeic acid (*16*). Such phenolics have not been thoroughly investigated for their ability to modulate oxidative stress-induced cellular injury. In this study, we initially investigated the potential neuroprotective effects of organic vs aqueous extracts of Champagne wine in order to gain an insight into which individual components hold the most neuroprotective potential. These major polyphenolic constituents, notably tyrosol, caffeic acid, and gallic acid, were then investigated for their ability to protect against peroxynitrite-induced neuronal injury.

MATERIALS AND METHODS

Chemical and Reagents. Sodium nitrite, manganese dioxide, hydrogen peroxide solution (30%, wt/vol), gallic acid, *p*-coumaric acid, and caffeic acid were from Sigma Chemical Co. (Poole, United

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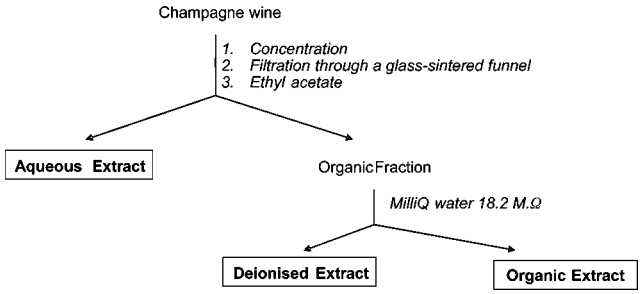

Figure 1. Champagne wine extraction procedure. Bottles of Champagnes were degassed, concentrated under vacuum, and extracted with ethyl acetate. While the aqueous solution obtained was concentrated under reduced pressure, the intermediate organic solution was washed with MilliQ water 18.2 MΩ cm to obtain two brand new fractions: organic and MilliQ, respectively. All of the concentrates were then frozen and stored at −80 °C until use.

Kingdom). Tyrosol was from Extrasynthese (Lyon, France). Caftaric acid was from Apin Chemicals (Abingdon, United Kingdom). Synthesis of ethanolic ester of caffeic acid was performed as previously described (*17*). Alamar blue reagent was from Serotec (Oxford, United Kingdom), and 3-morpholinosydnonimine (SIN-1) was from Alexis Chemical (Nottingham, United Kingdom). Solvents (methanol, acetonitrile, and ethanol) were all of high-performance liquid chromatography (HPLC) grade and were purchased from Fisher Scientific (Loughborough, United Kingdom). Ethyl acetate was purified by distillation on a Raschig column. When necessary, compounds were prepared using ultrapure water 18.2 MΩ cm (Purite Ltd., Oxon, United Kingdom).

Preparation of Champagne Wine Extracts. Champagne extracts were prepared using Champagne wine derived from both Chardonnay and Pinot Noir and Pinot Meunier grapes as reported previously (*18*). Briefly, Champagne was degassed and concentrated under vacuum, and a 600 mL volume was filtered through a glass-sintered funnel (volume, 500 mL; diameter, 95 mm; and porosity, 4) and extracted three times with 800 mL of ethyl acetate (**Figure 1**). The aqueous extract (AQ) obtained was concentrated under reduced pressure at a temperature below 40 °C with an R-200 rotary evaporator (Buchi, Rungis, France) to yield a dark-brown concentrate (yield = 15.47 g/L). The organic extract was also concentrated under vacuum (12-fold concentration) and then washed three times with 300 mL of MilliQ water 18.2 MΩ cm. The resulting organic fraction (O) was dried with Na_2SO_4 and concentrated under reduced pressure to yield a brown aromatic residue (yield = 94.4 mg/L). The MilliQ fraction (M_Q) was also concentrated under reduced pressure to give a bright brown residue (yield = 1.56 g/L). All of the concentrates were stored at −80 °C until use.

Analysis of Champagne Wine Extracts for Phenolic Content. HPLC analysis was performed using an Agilent 1100 Series linked to a diode array detector. Champagne wine extracts (5 mg mL^{-1}) were separated using a C18 Nova Pak column (250 mm × 4.6 mm i.d., 5 μm particle size), fitted with a guard column C18 NovaPak (Waters Ltd., Elstree, United Kingdom). The mobile phase consisted of A, 5% methanol + 5 N hydrogen chloride (0.1%) in water, and B, 5 N hydrogen chloride (0.1%) in acetonitrile−methanol (1:1), and was pumped through the column at 0.7 mL min^{-1}. Samples (50 μL) were injected and separated using the following gradient system (min/% B): 0/5, 5/5, 40/50, 55/100, 59.9/100, and 60/5 for detection of all compounds. The eluant was monitored by photodiode array detection at 254, 280, 320, and 370 nm, and spectra of products were obtained over the 220−600 nm range. All data were analyzed using ChemStation software.

Peroxynitrite Reaction with the Organic Extract of Champagne Wine. The reaction between Champagne wine extracts and peroxynitrite was investigated. Peroxynitrite was prepared using a stopped flow apparatus as previously described (*19*, *20*). Briefly, acidified hydrogen peroxide (20 mL of 1 M H_2O_2 in 0.7 M HCl, final concentration) was rapidly mixed with sodium nitrite (20 mL of 0.2 M $NaNO_2$, final concentration), and the reaction was quenched using ice-cold potassium hydroxide (1.5 M in 40 mL) to form peroxynitrite ($ONOO^-$). Manganese dioxide was used to remove unreacted hydrogen peroxide. The solution was filtered, and the peroxynitrite concentration was determined from its UV absorbance at 302 nm on an Ultraspec 1100 Pro spectrophotometer (Amersham Pharmacia Biotech) using ϵ_{302} = 1670 M^{-1} cm^{-1} (*21*).

Organic crude extract of Champagne wine (final concentration, 5 mg mL^{-1}) was reacted by the bolus addition of peroxynitrite (final concentration, 250 and 500 μM). The reaction mixture was stirred for 30 s at room temperature before injection in HPLC. Analytical separation used was the same as described above.

Primary Cortical Neurons Culture. Primary cultures of mouse cortical neurons were prepared from 14 to 16 day old Swiss mouse embryos (NIH, Harlan, United Kingdom). Briefly, cortices were isolated by dissecting out the striata and removing the meninges under a stereomicroscope. Cortices were then mechanically dissociated, using a fire-polished glass Pasteur pipet in phosphate-buffered saline (PBS, Ca^{2+}- and Mg^{2+}-free) supplemented with glucose (33 mM). Cells were plated into 16 mm multiwell plates that had been coated overnight with 15 μg/mL poly-L-ornithine (Sigma) and then with culture medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Paisley, United Kingdom) for 2 h. After removal of the final coating solution, cells were seeded (10^6 cells/mL) in a serum-free medium composed of a mixture of Dulbecco's modified Eagle medium and F-12 nutrient (1:1 v/v) (Gibco) supplemented with glucose (33 mM), glutamine (2 mM), sodium bicarbonate (6.5 mM), HEPES buffer (pH 7.4, 5 mM), streptomycin (100 μg/mL), and penicillin (100 IU/mL). A mixture of hormones and salts composed of insulin (25 μg/mL), transferrin (100 μg/mL), putrescine (60 μg/mL), progesterone (20 nM), and sodium selenate (30 nM) (all from Sigma) was also added to the cell culture medium. The cells were cultured at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 and were used after 5−6 days in vitro when the majority of cells were neuronal and there were <2% detectable glial elements, as determined by the lack of glial fibrillarly acidic protein (GFAP) immunoreactivity.

Assessment of Neuronal Damage in Presence of SIN-1. The cytotoxicity of peroxynitrite ($ONOO^-$) on neuronal cells was assessed using the peroxynitrite generator, SIN-1, which generates $ONOO^-$ at a rate of ∼1 μM s^{-1} (*22*). Prior to SIN-1 exposure, the neuronal growth medium was removed and retained at 37 °C and 5% CO_2. Neurons were exposed to vehicle or freshly prepared SIN-1 at different concentrations (500−2000 μM; 2 h) in HEPES-buffered incubation medium (HBM): 140 mM NaCl, 5 mM KCl, 5 mM $NaHCO_3$, 1.2 mM Na_2HPO_4, 1.2 mM $CaCl_2$, 5.5 mM glucose, and 20 mM HEPES, pH 7.4. Following exposure, the HBM buffer was removed and neurons were reincubated with the original conditioned neuronal growth medium for 24 h prior to assessment of neuronal damage using the Alamar Blue assay. Alamar Blue solution (10% v/v) was added to each well, and plates were returned to the incubator for 2−3 h, before fluorescence was measured (Ex = 540 nm; Em = 612 nm) on a Tecan GENios multiplate reader (Tecan GENios, Theale, United Kingdom).

Protective Effect of Phenolic Compounds and Champagne Wine Extracts. To assess the neurotoxicity of Champagne wine polyphenols, neurons were exposed to tyrosol, caffeic acid, and gallic acid (0.1−50 μM) or aqueous, MilliQ, and organic Champagne extracts (0.1−50 μg/mL) for 24 h, and Alamar Blue assays were performed to assess damage. To assess the potential protective effects of Champagne wine polyphenols, cortical neurons were pretreated for 18 h with phenolic compounds: tyrosol, caffeic acid, or gallic acid (0.01−10 μM) or aqueous, MilliQ, and organic extracts (0.1 or 1 μg/mL). After 18 h, medium was removed and retained and neurons were washed with HBM prior to the addition of HBM buffer containing SIN-1 (500 μM; 2 h). Following SIN-1 exposure, HBM buffer was removed and replaced with conditioned media for 24 h before assessment of neuronal injury. To prevent any direct scavenging effects, both Champagne extracts and phenolic compounds were removed and neurons were washed prior to SIN-1 addition.

Statistics. Data were expressed as means ± standard deviations (SDs). The statistical significance of the results was determined by one-way analysis of variance (ANOVA) followed by a posthoc *t* test where statistical significance was set at $p < 0.05$.

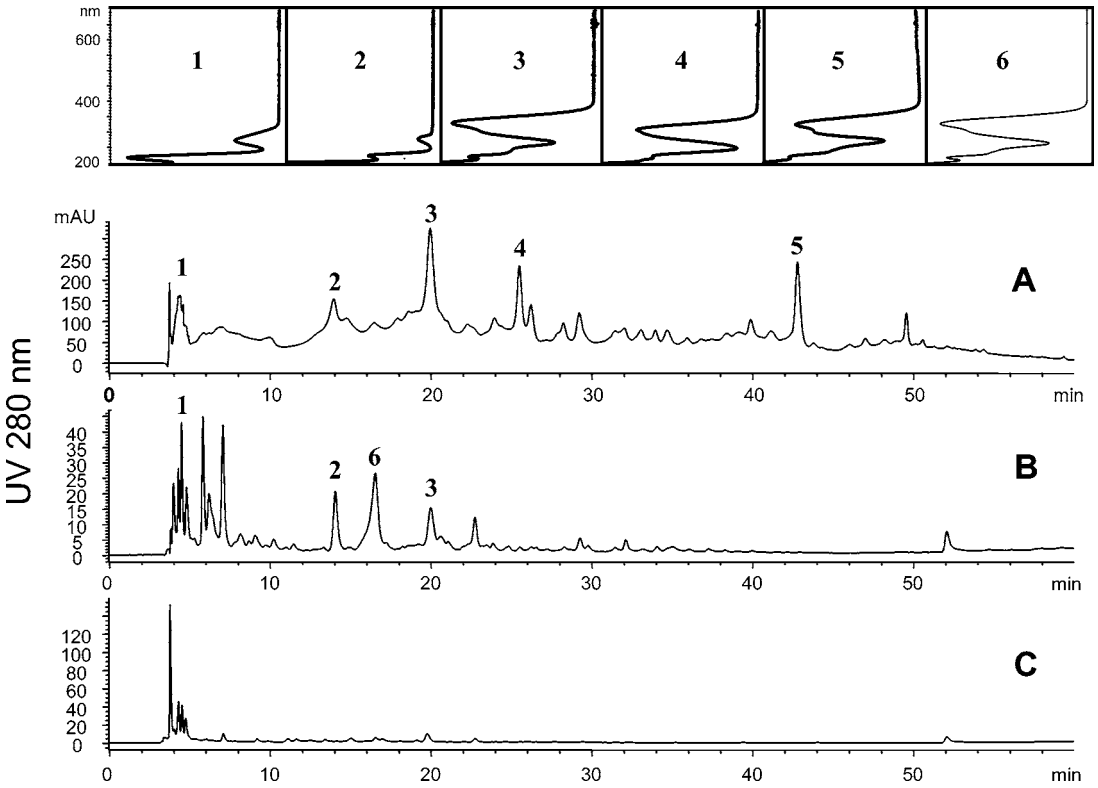

Figure 2. Chromatographic separation of organic (**A**), MilliQ (**B**), and aqueous (**C**) extracts of Champagne wine. Separation was carried out as described in the Materials and Methods. Key: 1, gallic acid; 2, tyrosol; 3, caffeic acid; 4, *p*-coumaric acid; 5, ethyl caffeate; and 6, caftaric acid.

RESULTS

Chromatographic Separation of Champagne Wine Extracts. The extraction procedure used for Champagne wine allowed us to prepare three separate fractions (**Figure 1**). Chromatographic separation of the organic, MilliQ, and aqueous extracts was achieved by reverse phase HPLC, and multiple peaks were detected in each extract at 280 nm (**Figure 2**). The chromatographic profiles of both the organic extract (**Figure 2A**) and the MilliQ extract (**Figure 2B**) revealed the presence of gallic acid (peak 1), tyrosol (peak 2), and caffeic acid (peak 3), which were characterized by their spectra, their retention time, and by comparison with authentic phenolic standards. The organic extract (**Figure 2A**) also contained *p*-coumaric acid (peak 4) and ethyl caffeate (peak 5). However, none of these latter compounds within the MilliQ extract were detected, which was observed to alternatively contain caftaric acid (peak 6). Although there were many smaller peaks present in both organic and MilliQ extracts, we were able to identify the major constituents. As expected, the aqueous extract (**Figure 2C**) consisted of mainly polar components.

Peroxynitrite Reaction with Champagne Wine Extracts. Reactions between the peroxynitrite and the crude organic extract of Champagne wine were carried out. Reacting the organic extract with 250 μM peroxynitrite (**Figure 3B**) resulted in the disappearance of caffeic acid (peak 3) as compared to the control (**Figure 3A**). In these conditions, ethyl caffeate (peak 5) was observed to be slightly decreased while *p*-coumaric acid (peak 4) was not observed to be modified. Moreover, during the course of our experiment, we observed an increase in tyrosol (peak 2) and gallic acid (peak 1). Although we did not carry out any other experiments in order to identify more accurately the reasons of this modification, we think that it is more likely to be the result of metabolite cleavages, releasing aglycone forms within the reaction mixture. Raising the amount of peroxynitrite up to 500 μM (**Figure 3C**) resulted in the complete disappearance of caffeic acid (peak 3), followed by ethyl caffeate (peak 5). Tyrosol seemed not to react to peroxynitrite addition, as no decrease was observed during the course of our experiments. As observed previously, no modification was detected for *p*-coumaric acid (peak 4).

Assessment of Neuronal Damage Induced by Peroxynitrite. SIN-1 was used to assess the toxicity of peroxynitrite on primary cortical neurons. The exposure of primary cortical neuronal cell cultures to SIN-1 (0.5−2.0 mM, 2 h) resulted in concentration-dependent neuronal injury (**Figure 4**). Exposure to neurons to SIN-1 (0.5 mM) caused significant neuronal damage as was evidenced by a fall in the ability of neurons to metabolize Alamar Blue (52 $\pm$ 3.26%; $p < 0.001$). Higher concentrations (2 mM SIN-1) resulted in increased levels of neuronal death (70 $\pm$ 1.51%; $p < 0.001$), although subsequent protection experiments utilized SIN-1 at a concentration of 0.5 mM in order to achieve approximately 50% neuronal injury.

Ability of Champagne Wine Extracts and Phenolics To Protect Primary Neurons against Peroxynitrite-Induced Toxicity. To investigate the potential neuroprotective effects of Champagne wine polyphenols against peroxynitrite-induced toxicity, we initially carried out control experiments to assess their neurotoxicity. Neuronal viability remained unchanged in the presence of aqueous, MilliQ, or organic extracts at concentrations up to 50 μg/mL. In addition, tyrosol and caffeic acid and gallic acid did not induce any significant neuronal toxicity at concentrations below 10 μM.

Neuronal cultures were exposed to SIN-1 with or without pretreatment with Champagne extracts (0.1−1 μg/mL) or phenolic compounds (0.01−10 μM). Pretreatment with Champagne wine extracts (0.1 or 1 μg/mL) resulted in significant protection against SIN-1-induced neurotoxicity (**Figure 5A**−**C**). At low concentrations (0.1 μg/mL), the organic extract (**Figure 5A**) was observed to exert the greatest protective effect, increasing neuronal viability from 49 $\pm$ 2.65 to 72 $\pm$ 6%, as

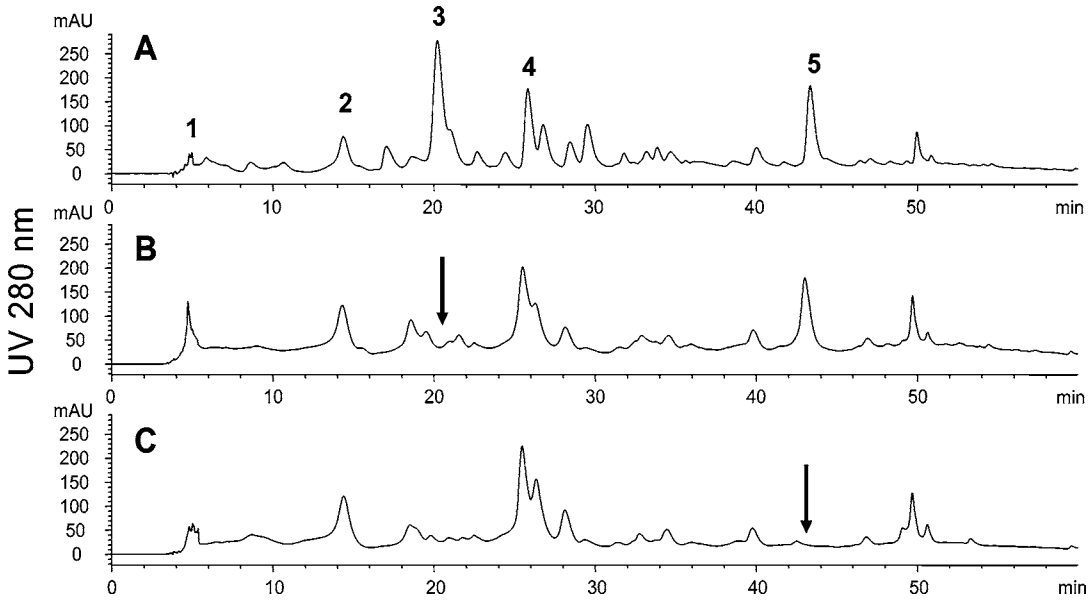

Figure 3. Peroxynitrite reaction with organic Champagne wine extract. Organic crude extract of Champagne wine (**A**) was reacted with peroxynitrite 250 (**B**) and 500 μM (**C**) for 30 s before injection in HPLC. Separation was carried out as described in the Materials and Methods. Key: 1, gallic acid; 2, tyrosol; 3, caffeic acid; 4, *p*-coumaric acid; and 5, ethyl caffeate. Black arrows indicate the disappearance of compounds after reaction with peroxynitrite.

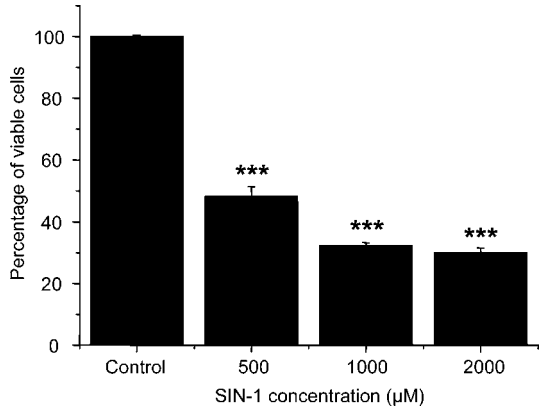

Figure 4. SIN-1 induces toxicity on primary cortical neurons. Five to six DIV primary neurons were exposed to freshly prepared SIN-1 (500–2000 μM) or vehicle for 2 h. After 24 h, cell viability was determined by Alamar blue reduction. Bars represent the mean ± SD of quadruplicate wells from single experiments repeated three times with similar results (***$p <$ 0.001 as compared with control and as analyzed by one-way ANOVA followed by posthoc *t*-test).

compared to 66 ± 2 and 68 ± 10% for MilliQ (**Figure 5B**) and aqueous extracts (**Figure 5C**), respectively. Concentration-dependent neuroprotective effects were observed when cortical neurons were pretreated with the polyphenols, tyrosol, caffeic acid, or gallic acid (**Figure 6**). Hundred nanomolar concentrations were able to protect primary neurons against peroxynitrite-induced toxicity, restoring cellular viability from 49 ± 2.65 to 61 ± 1.5% for tyrosol (**Figure 6A**), 66 ± 4% for caffeic acid (**Figure 6B**), and 65 ± 4.8% for gallic acid (**Figure 6C**) ($p <$ 0.05), with higher concentrations (10 μM) completely restoring neuronal viability.

DISCUSSION

Red wine consumption has been linked to a lower incidence of cardiovascular disease and has been suggested to counteract the onset of neurological disorders, such as Alzheimer's disease and dementia (*23*). The ability of wine to counteract disease progression is thought to be due to its high content of polyphenols, such as flavonoids and phenolic acids (*24*). However, although white wine has significantly lower concentrations of these photochemicals, Champagne has been shown to be relatively rich in polyphenols such as hydroxybenzoic acids, hydroxycinnamic acids (and their tartaric derivative esters), flavonoids, phenolic alcohols, and phenolic aldehydes (*16*). This is because unlike white wine, Champagne is made is using three grape varieties: Chardonnay and two red grapes, Pinot Noir and Pinot Meunier. There are two distinct varieties produced as follows: "Blanc de Blancs" champagne (BB), which is made exclusively with Chardonnay grapes, and "Blanc de Noirs" champagne (BN), which is made from two red grape varieties. Most Champagne is a blend of these two forms with most of the polyphenol content deriving from the latter.

To assess the polyphenol content of Champagne wine and to understand which polyphenols were responsible for potential neuroprotective effects, three individual extracts were generated. These extracts were derived from a mixture of BB and BN Champagne wines, and our extraction procedure led to the isolation of three distinct extracts, organic and MilliQ, which were rich in phenolics such as caffeic acid, tyrosol, and caftaric acid, and an aqueous extract, which had no detectable phenolics but was rich in polar components. HPLC analysis of the extracts allowed characterization of individual phenolic components that may be responsible for cellular actions. Even though concentration and composition of phenolic compounds may vary with variety, vintage, and a wide range of environmental factors (*25*), we were able to identify the major phenolic constituents present in Champagne wine.

All three extracts of Champagne wine exerted protection against peroxynitrite-induced neuronal injury, although the organic extract was observed to offer the greatest degree of protection at the lowest exposure concentration. The ability to protect neurons against peroxynitrite-induced injury is relevant as peroxynitrite has been implicated to be a mediating toxic agent in a wide array of neurodegenerative disorders (*26*). In particular, peroxynitrite may be formed during neuroinflammation and may lead to cytotoxic actions (*27*) via its ability to oxidize and nitrate proteins, lipids, and DNA (*28*). For example, 3-nitrotyrosine, a product of peroxynitrite attack on tyrosine,

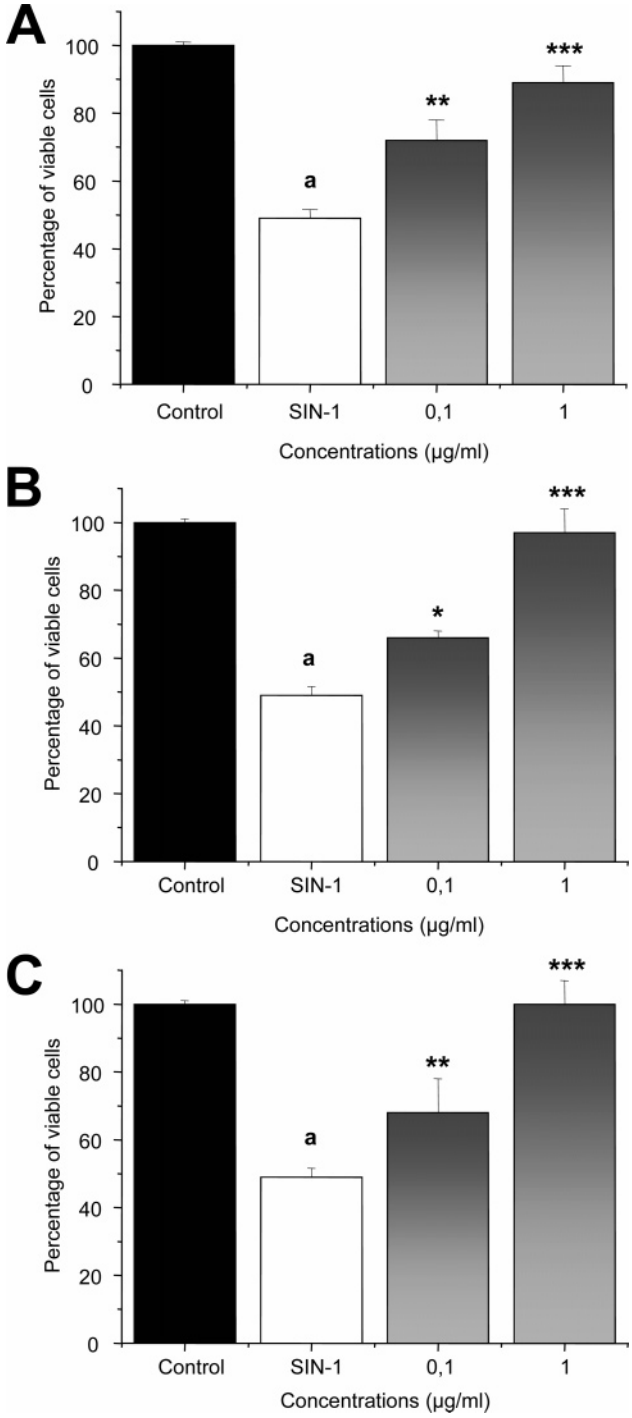

Figure 5. Champagne wine extracts attenuate SIN-1-induced neuronal death of primary neurons. Following pretreatment of 0.1–1 μg/mL Champagne wine extracts (**A**, organic extract; **B**, MilliQ extract; and **C**, aqueous extract) for 18 h, 5–6 DIV primary neurons were exposed to SIN-1 (500 μM) for 2 h. Cell viability was determined after 24 h by Alamar blue reduction assay. Bars represent the mean ± SD of quadruplicate wells from single experiments repeated three times with similar results a, indicates decrease in viability compared to the control ($p < 0.001$); ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$ as compared with the cultures treated with SIN-1, as analyzed by one-way ANOVA followed by posthoc *t*-test).

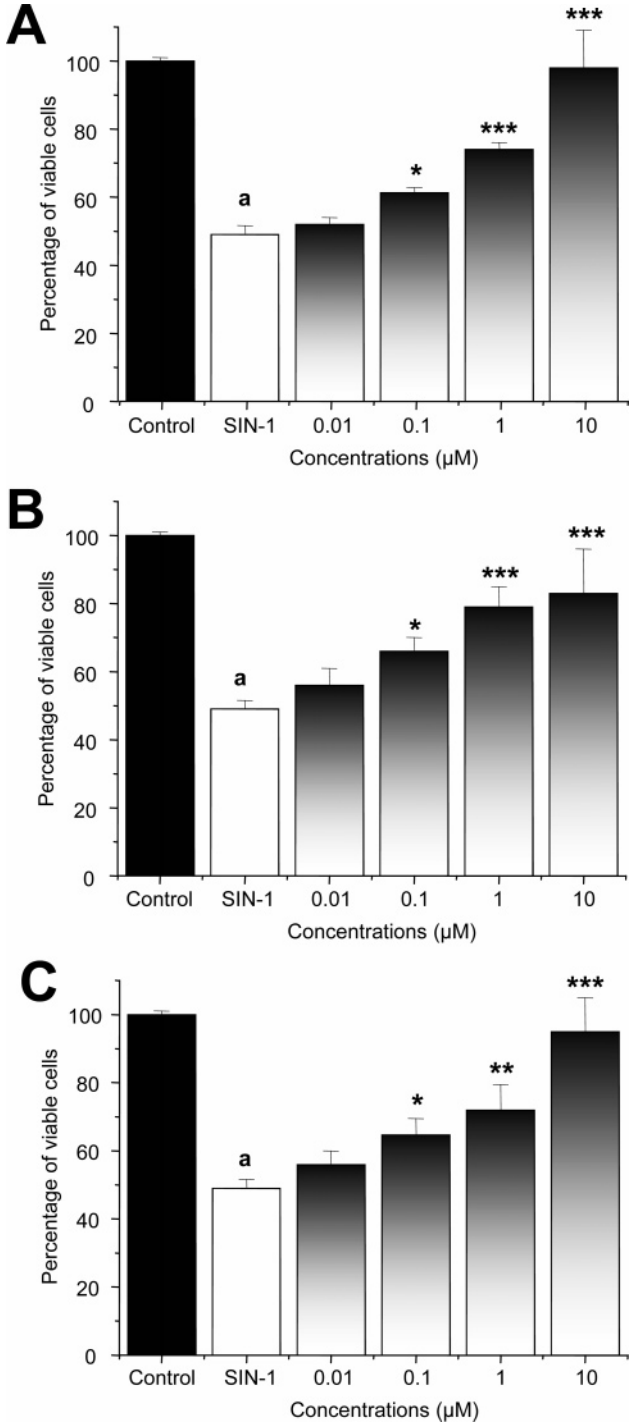

Figure 6. Effect of treatment with tyrosol (**A**), caffeic acid (**B**), and gallic acid (**C**) against toxicity induced by SIN-1 (500 μM). Following pretreatment with tyrosol, caffeic acid, and gallic acid (0.01–10 μM) for 18 h, 5–6 DIV primary neurons were exposed to SIN-1 (500 μM) for 2 h. Cell viability was determined after 24 h by Alamar blue reduction assay. Bars represent the mean ± SD of quadruplicate wells from single experiments repeated three times with similar results a, indicates decrease in viability compared to the control ($p < 0.001$); ***$p < 0.001$, ** $p < 0.01$, and *$p < 0.05$ as compared with the cultures treated with SIN-1 and as analyzed by one-way ANOVA followed by posthoc *t*-test).

has been found to be localized to Lewy bodies within melanized neurons and in amorphous deposits associated with intact and degenerating dopaminergic cells (*7*). The organic and MilliQ extracts were observed to exert strong protective effects against peroxynitrite-induced neuronal damage. These fractions were observed to contain the majority of phenolic components present in Champagne wine, notably tyrosol, gallic acid, caffeic acid, and caftaric acid. Such phenolics have previously been reported to evoke cellular protection against oxidative injury. For example, hydroxycinnamates, hydroxybenzoic acids, and phe-

nolics alcohols have shown to exert health benefits by exhibiting both antioxidant and anti-inflammatory properties in vitro (*29*). Furthermore, caffeic acid and tyrosol have been shown to regulate inflammatory responses by modulating both oxidative stress and inflammatory reactions (*30*). Direct scavenging of $ONOO^-$ by dietary phenolics may represent one mechanism by which these compounds may exert their beneficial actions in vivo. It is likely that these compounds react with peroxynitrite either by directing the nitration to their own structures, in the case of monohydroxylated compounds such as *p*-coumaric acid, or by deactivating $ONOO^-$ by electron donation (*31*). In our experiments, reacting peroxynitrite with organic Champagne wine extract did not yield to any modification of *p*-coumaric acid levels. However, a strong decrease of caffeic acid and its derivative was noted, implicating a potential direct scavenging of peroxynitrite leading to *o*-quinone formation.

To assess which phenolics were contributing directly to the protection against peroxynitrite injury, we also tested the major phenolics present in the organic and MilliQ extracts in our neuronal model. The quantities of tyrosol, caffeic, and gallic acid in a glass of champagne wine are in the region of 22, 1.2, and 0.4 μM, respectively. Native phytochemicals in the diet are usually subjected to extensive metabolism following oral ingestion. In the upper gastrointestinal tract, dietary phytochemicals act as substrates for a number of enzymes and are subjected to extensive metabolism by β-glucosidase enzymes, phase I enzymes (hydrolyzing and oxidizing), such as cytochrome P450, and phase II enzymes (conjugating and detoxifying) found in both the small intestine and the liver. Following absorption and metabolism, the circulating amounts of hydroxycinnamates and other phenolic acids (both free and conjugated forms) have been shown to be present in the nanomolar to low micromolar range (*32*). For example, tyrosol was observed to be glucuronidated following absorption, metabolism, and microflora-dependent transformation (*33*). Similar levels of caffeic acid were found in plasma in a concentration-dependent manner on red wine intake while gallic acid was observed as the most well-absorbed polyphenol (*34*). Although very few studies have addressed the metabolic pathways of these compounds in humans after dietary consumption, such small phenolics are likely to access the brain via transfer across the blood−brain barrier (BBB) on amino acid transporters. For example, caffeic acid shares structural similarities with L-DOPA and may be transported across the BBB via catecholaminergic receptors. All of the phenolics tested protected against neuronal injury at nanomolar concentrations, with the greatest degree of protection observed with tyrosol and caffeic acid. Caffeic acid was also protective; although at higher concentrations (10 μM), its protective ability was lower than that of tyrosol and gallic acid possibly due to the potential formation of intracellular *o*-quinone species from this catechol, which could lead to the rapid depletion of intracellular thiols such as glutathione (*35*). We did not assess the protective actions of caftaric acid, as although it is present in high concentrations in Champagne wine, it is more likely to be cleaved to caffeic acid by the action of esterases present in the gastrointestinal tract (*36*, *37*). In addition, although the aqueous extract did not contain any major phenolics, it also evoked significant neuroprotection against oxidative neuronal damage. This suggests that other, more water-soluble, components must be present in this fraction, which also influence the beneficial potential of Champagne wine. Future studies will be directed at identifying such components and their individual neuroprotective effects.

The ability of the phenolic acids to exert cytoprotective actions against oxidative insults has been proposed to be linked to their antioxidant activity. However, we observed that their ability to protect against peroxynitrite-mediated neurotoxicity occurs at concentrations that are unlikely to scavenge significant amounts of peroxynitrite intracellularly. Indeed, previous investigations have reported that direct reaction of caffeic acid and other hydroxycinnamic acids with $ONOO^-$ occurs at micromolar concentrations (*38*). Furthermore, we have recently reported that while the flavonoid hesperetin protects fibroblasts against peroxynitrite-induced injury, its ability to react directly with peroxynitrite occurs with an IC_{50} of 12.3 μM (*39*). Therefore, it is likely that phenolics such as tyrosol, caffeic acid, and gallic acid exert protection via their ability to modulate neuronal signaling pathways, as has been reported for other polyphenols such as the flavonoids (*40*). Flavonoids have been shown to exert cytoprotective effects against oxidative injury via their potential to interact within the MAP kinase signaling pathway (*41*) and the PI3 kinase/Akt signaling cascade. Activation of these pathways controls cellular responses to oxidative stress, and it is probable that phenolic acids such as tyrosol and caffeic acid may act in a similar manner. Future investigations will be aimed at investigating whether Champagne wine polyphenols, such as tyrosol and caffeic acid, as well as other as yet unidentified components, are able to protect against oxidative injury through the activation of survival signaling or inhibition of proapoptotic signaling in neurons.

In summary, we have demonstrated that both Champagne wine extracts and phenolic constituents are able to protect primary cortical neurons against peroxynitrite-induced toxicity. The presence of phenolic components such as caffeic acid, tyrosol, and gallic acid in Champagne wine may underlie these protective effects. Future investigations will focus on determining the mechanism by which these phenolic acids exert neuromodulatory activities.

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