

# Neuroprotective effects of naturally occurring polyphenols on quinolinic acid-induced excitotoxicity in human neurons

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## Keywords

Alzheimer's disease; excitotoxicity; NAD<sup>+</sup>; polyphenols; quinolinic acid

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Quinolinic acid (QUIN) excitotoxicity is mediated by elevated intracellular Ca<sup>2+</sup> levels, and nitric oxide-mediated oxidative stress, resulting in DNA damage, poly(ADP-ribose) polymerase (PARP) activation, NAD<sup>+</sup> depletion and cell death. We evaluated the effect of a series of polyphenolic compounds [i.e. epigallocatechin gallate (EPCG), catechin hydrate, curcumin, apigenin, naringenin and gallotannin] with antioxidant properties on QUIN-induced excitotoxicity on primary cultures of human neurons. We showed that the polyphenols, EPCG, catechin hydrate and curcumin can attenuate QUIN-induced excitotoxicity to a greater extent than apigenin, naringenin and gallotannin. Both EPCG and curcumin were able to attenuate QUIN-induced Ca<sup>2+</sup> influx and neuronal nitric oxide synthase (nNOS) activity to a greater extent compared with apigenin, naringenin and gallotannin. Although Ca<sup>2+</sup> influx was not attenuated by catechin hydrate, nNOS activity was reduced, probably through direct inhibition of the enzyme. All polyphenols reduced the oxidative effects of increased nitric oxide production, thereby reducing the formation of 3-nitrotyrosine and poly (ADP-ribose) polymerase activity and, hence, preventing NAD<sup>+</sup> depletion and cell death. In addition to the well-known antioxidant properties of these natural phytochemicals, the inhibitory effect of some of these compounds on specific excitotoxic processes, such as Ca<sup>2+</sup> influx, provides additional evidence for the beneficial health effects of polyphenols in excitable tissue, particularly within the central nervous system.

## Introduction

Quinolinic acid (QUIN) cytotoxicity is known to be involved in the pathogenesis of several central nervous system disorders, including Alzheimer's disease (AD) [1–3], amyotrophic lateral sclerosis [4], Huntington's disease [5] and the AIDS dementia complex [6]. We

have previously shown that the *N*-methyl-D-aspartic acid (NMDA) receptor can be activated by pathophysiological concentrations of QUIN in both human astrocytes and neurons, rendering these cells susceptible to injury via an excitotoxic process [7]. Excitotoxic-

## Abbreviations

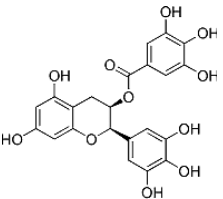
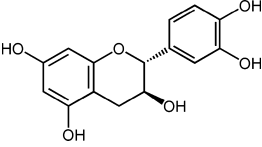
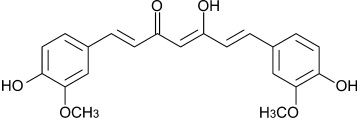
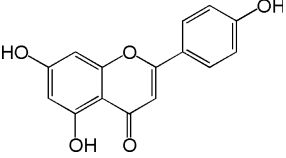
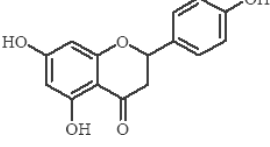
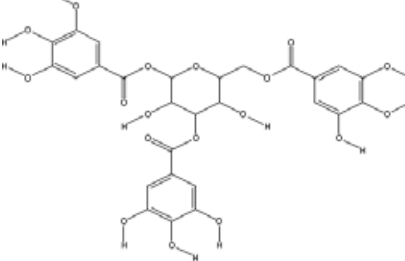
3-NT, 3-nitrotyrosine; AD, Alzheimer's disease; EPCG, epigallocatechin gallate; iNOS, inducible nitric oxide synthase; LDH, lactate dehydrogenase; NMDA, *N*-methyl-D-aspartic acid; nNOS, neuronal nitric oxide synthase; NO•, nitric oxide; PAR, poly(ADP-ribose); PARP, poly(ADP-ribose) polymerase; QUIN, quinolinic acid; RNS, reactive nitrogen species; ROS, reactive oxygen species.

ity can occur through over-activation of the NMDA receptor, with subsequent influx of  $\text{Ca}^{2+}$ , activation of both neuronal nitric oxide synthase (nNOS) and inducible nitric oxide synthase (iNOS), and excess generation of nitric oxide ( $\text{NO}\bullet$ ) [8].

$\text{NO}\bullet$  is a potent vasodilator and an important neurotransmitter that is not considered toxic at physiological concentrations [9]. However, the  $\text{NO}\bullet$  radical is largely unstable in the cellular system, and can react via complex pathways to yield tertiary reactive nitrogen species (RNS), such as  $\text{NO}_2^-$  and the peroxy nitrite free radical [10]. These molecules can cause DNA damage leading to activation of the nuclear DNA nick sensing enzyme poly(ADP-ribose) polymerase-1 (PARP-1) [11]. Activated PARP-1 synthesizes ADP-ribose polymers from  $\text{NAD}^+$  [11]. Over-activation of PARP-1 can lead to the depletion of intracellular  $\text{NAD}^+$  and ATP stores, leading to a number of deleterious processes, including mitochondrial permeability [12], overproduction of superoxide [12] and the release of cell death mediators [11]. We have previously shown that QUIN can induce PARP activation and subsequent  $\text{NAD}^+$  depletion and cell death in primary human neurons at pathophysiological concentrations [7]. Therefore, strategies directed at reducing QUIN-induced  $\text{NO}\bullet$  production and free radical damage may prove beneficial in treatments of neurodegenerative disease.

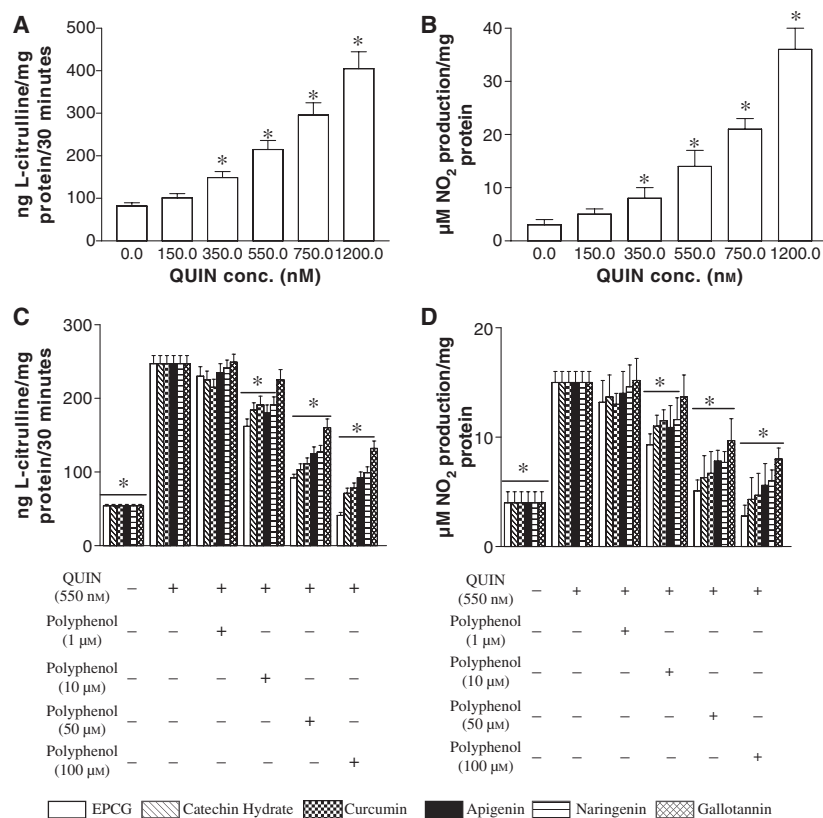
Extensive investigations have been undertaken to determine the neuroprotective effect of polyphenolic-rich beverages, such as teas and red wine [13–16]. Several neuroprotective mechanisms of action have been proposed, including antioxidant and/or anti-inflammatory properties [17]. Studies have shown that frequent consumption of fruit and vegetable juices, which are high in polyphenols, are associated with a substantially decreased risk of AD [18]. The Kame Project found that subjects who reported drinking juices three or more times per week were 76% less likely to develop signs of AD than those who drank less than one serving per week. Even drinking juices once or twice a week was found to reduce the risk by 16% [18]. Numerous studies have shown that green tea polyphenols can protect against excitotoxicity in neuronal cells, although the exact mechanism remains unclear [19]. Tea consumption *ad libitum* by rodents was shown to afford neuroprotection against oxidative damage in normal aging [20], and through combination with the NMDA channel blocker memantine against brain excitotoxicity [21]. Some studies have shown that tea- and wine-derived catechins, in parallel with the individual flavonol quercetin, can reduce the concentrations of increased reactive oxygen species

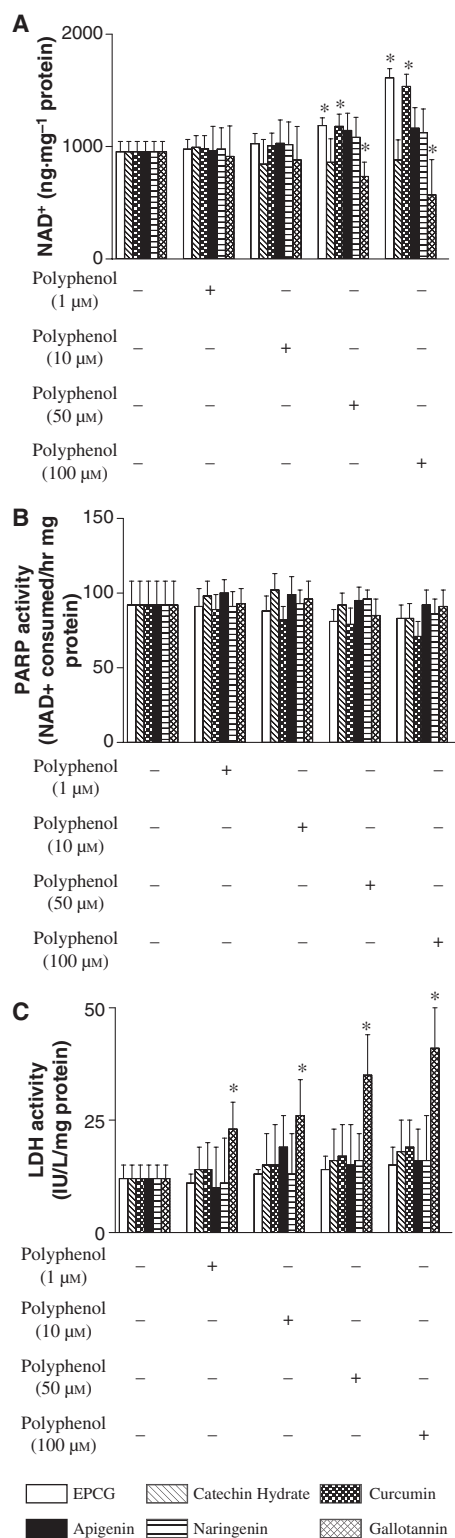
**Table 1.** Structure of the green tea polyphenols used in the present study.

Polyphenol	Chemical structure
EPCG	
Catechin hydrate	
Curcumin	
Apigenin	
Naringenin	
Gallotannin	

(ROS) and RNS [22–25] and intracellular  $\text{Ca}^{2+}$  levels in the synapse [26]. Other studies have indicated a significant inhibitory effect of catechins and apigenin upon iNOS activity [27,28]. However, to our knowledge, no study has reported the potential inhibitory effect of naturally occurring polyphenolic compounds on nNOS activity and intracellular  $\text{Ca}^{2+}$  influx in human neurons following exposure to pathophysiological concentrations of QUIN.

In the present study we evaluated the potential inhibitory effect of several polyphenolic compounds present in green tea, namely epigallocatechin gallate (EPCG),





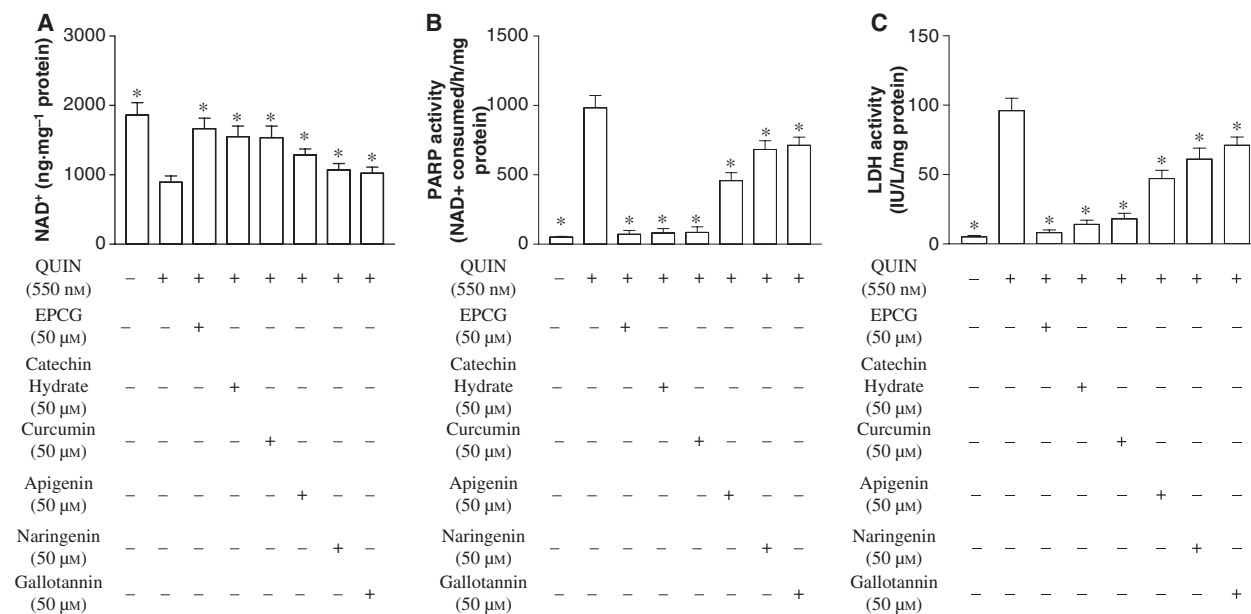
rons after 24 h of treatment. Treatment with EPCG and curcumin significantly increased intracellular NAD<sup>+</sup> levels in a dose-dependent manner (Fig. 2A), but no significant difference was observed for PARP

**Fig. 2.** Effect of polyphenols on intracellular NAD<sup>+</sup> levels, PARP activation and cell death in human neurons. Effect of: (A) EPCG, catechin hydrate, curcumin, apigenin, naringenin and gallotannin on intracellular NAD<sup>+</sup> levels for 24 h (\**P* < 0.05 compared with medium alone); (B) EPCG, catechin hydrate, curcumin, apigenin, naringenin and gallotannin on PARP activity for 1 h (\**P* < 0.05 compared with medium alone); (C) EPCG, catechin hydrate, curcumin, apigenin, naringenin and gallotannin on extracellular LDH activity (\**P* < 0.05 compared with medium alone); *n* = 3 for each treatment group.

(Fig. 2B) and LDH activities (Fig. 2C). On the contrary, gallotannin induced a dose-dependent decrease in intracellular NAD<sup>+</sup> levels (Fig. 2A) and a dose-dependent increase in extracellular LDH activity (Fig. 2C). No significant difference was observed for PARP activity (Fig. 2B). Similarly, no significant differences were observed in intracellular NAD<sup>+</sup> levels (Fig. 2A), PARP (Fig. 2B) and extracellular LDH activities (Fig. 2C) for apigenin and naringenin.

#### Effect of EPCG, catechin hydrate, curcumin, apigenin, naringenin and gallotannin on QUIN-mediated NAD<sup>+</sup> depletion, extracellular LDH and PARP activation in human neurons

To assess the effects of polyphenols on QUIN-mediated NAD<sup>+</sup> depletion, PARP activation and extracellular LDH release (cell death), we measured intracellular NAD<sup>+</sup> levels, PARP and extracellular LDH activities in human neurons after 24 h of treatment. The addition of EPCG, catechin hydrate and curcumin (50 μM) significantly attenuated QUIN-mediated NAD<sup>+</sup> depletion after 24 h (Fig. 3A). Apigenin, naringenin and gallotannin also prevented NAD<sup>+</sup> depletion at the same concentration (50 μM), but to a lesser extent (Fig. 3A). As previously shown, neurons treated with QUIN at 550 nM for 1 h had significantly increased PARP activity compared with the control (Fig. 3B). Concomitant treatment of these cells with EPCG, catechin hydrate and curcumin (50 μM) significantly reduced PARP activity compared with QUIN treatment alone. Treatment with apigenin, naringenin and gallotannin (50 μM) also reduced PARP activity, but to a significantly lower degree than EPCG, catechin hydrate or curcumin (Fig. 3B). These results closely correlate with results presented for NAD<sup>+</sup> (Fig. 3A). Neurons treated with QUIN (550 nM) in the presence of selected polyphenols (50 μM) showed significantly reduced evidence of cell death as measured by extracellular LDH activity in culture supernatants after 24 h (Fig. 3C). Extracellular LDH activity was significantly reduced in the presence of EPCG, cate-



**Fig. 3.** Effect of polyphenols on QUIN-induced NAD depletion, PARP activation and cell death in human neurons. Effect of: (A) EPCG (50 μM), catechin hydrate (50 μM), curcumin (50 μM), apigenin (50 μM), naringenin (50 μM) and gallotannin (50 μM) on intracellular NAD<sup>+</sup> levels in the presence of QUIN (550 nM) for 24 h (\**P* < 0.05 compared with 550 nM QUIN alone); (B) EPCG (50 μM), catechin hydrate (50 μM), curcumin (50 μM), apigenin (50 μM), naringenin (50 μM) and gallotannin (50 μM) on PARP activity in the presence of QUIN (550 nM) for 1 h (\**P* < 0.05 compared with 550 nM QUIN alone); (C) EPCG (50 μM), catechin hydrate (50 μM), curcumin (50 μM), apigenin (50 μM), naringenin (50 μM) and gallotannin (50 μM) on extracellular LDH activity in the presence of QUIN (550 nM) (\**P* < 0.05 compared with 550 nM QUIN alone); *n* = 4 for each treatment group.

chin hydrate and curcumin compared with apigenin, naringenin and gallotannin (Fig. 3C). These results again directly correlate with data for NAD<sup>+</sup> depletion and PARP activity (Fig. 3A,B).

### QUIN induces intracellular Ca<sup>2+</sup> levels in cultured human neurons

Human fetal neurons were incubated with QUIN and a significant dose-dependent increase in intracellular Ca<sup>2+</sup> influx was observed (Fig. 4). As RNS were increased with increasing concentrations of QUIN (Fig. 1), it is reasonable to conclude that the formation of NO• is a downstream event in the QUIN-induced excitotoxic cascade mediated by Ca<sup>2+</sup> influx.

### Effect of EPCG, catechin hydrate, curcumin, apigenin, naringenin and gallotannin on QUIN-induced intracellular Ca<sup>2+</sup> in cultured human neurons

As mentioned above, QUIN stimulation induced a significant increase in intracellular Ca<sup>2+</sup>. Each of the polyphenols, EPCG, curcumin, apigenin, naringenin and gallotannin, significantly reduced intracellular Ca<sup>2+</sup> influx (Fig. 4). Attenuation of increased Ca<sup>2+</sup> influx was greatest with EPCG and curcumin

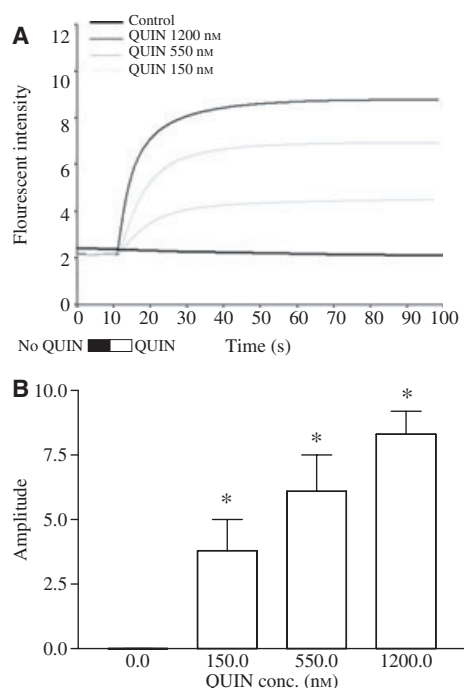
compared with apigenin and naringenin (Fig. 5). Interestingly, catechin hydrate did not ameliorate a QUIN-induced increase in intracellular Ca<sup>2+</sup> (Fig. 5).

### Detection of 3-nitrotyrosine (3-NT) formation in cultured human neurons

Immunocytochemistry was used to visualize protein nitration due to increased NO• production in cultured human neurons. Increased protein nitration in the form of increased 3-NT was observed in 20% of QUIN-treated cells compared with nontreated cells (Fig. 6A,B). Likewise, staining for 3-NT was less detectable in QUIN-treated neurons preincubated with EPCG (0%), catechin hydrate (0%) and curcumin (0%) compared with cells treated with apigenin (7%), naringenin (9%) and gallotannin (12%) (Fig. 6A,B).

### Detection of PAR expression in cultured human neurons

Immunocytochemistry studies were used to detect PAR formation following treatment with QUIN and selected polyphenols. The amount of PAR formed in living cells gives a direct indication of the extent of DNA damage. Higher immunoreactivity for PAR



**Fig. 4.** QUIN induces  $\text{Ca}^{2+}$  influx in human neurons. (A) Representative trace of intracellular  $\text{Ca}^{2+}$  induced by QUIN (150, 550 and 1200 nM). (B) Quantified amplitude of neuronal response to QUIN at the aforementioned concentrations (\* $P < 0.05$  compared with no QUIN);  $n = 4$  for each treatment group.

staining (25%) was detected in human neurons in the presence of QUIN (550 nM) compared with untreated cultures and cells cotreated with 50  $\mu\text{M}$  EPCG (4%), catechin hydrate (5%), curcumin (4%), apigenin (10%), naringenin (11%) and gallotannin (12%) for 1 h (Fig 7A,B). The presence of EPCG, catechin hydrate and curcumin in QUIN-exposed neurons resulted in the lowest PAR formation compared with cells treated with the other polyphenols (Fig. 7A,B). This indicates that the latter compounds exhibit a poorer neuroprotective effect against DNA damage compared with EPCG, catechin hydrate and curcumin.

## Discussion

The excitotoxin QUIN is one of the major end products of tryptophan catabolism in the central nervous system. Increased QUIN production by activated microglia/infiltrating macrophages has been reported in the brain in aging and in neuroinflammatory diseases [1]. For example, QUIN is found at high concentrations in immunoreactive amyloid plaques in the AD brain [1,2,29]. Given the complex aetiology and mechanisms of AD, QUIN probably plays a

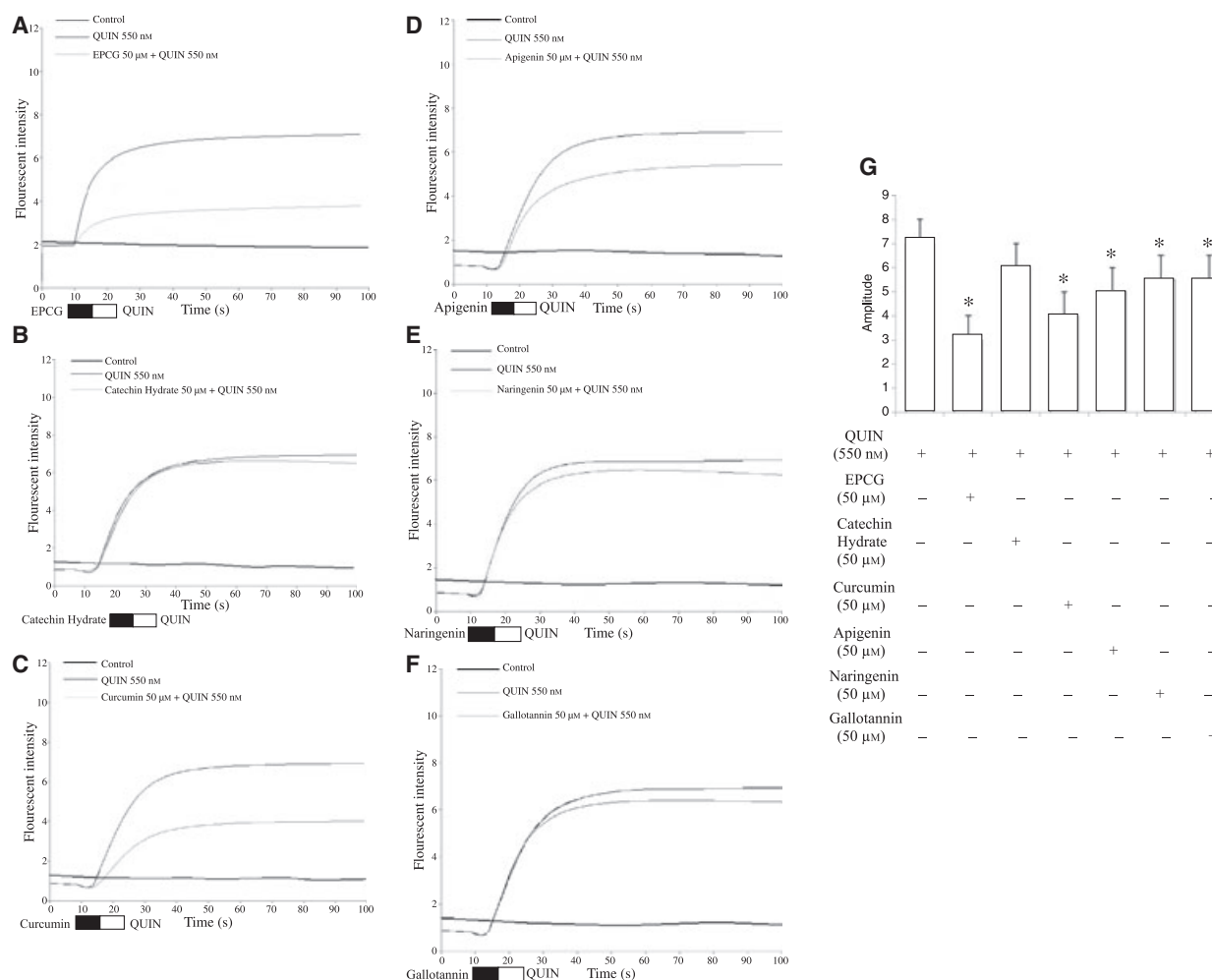
pivotal role in the neurodegenerative changes occurring in the brain [1,29,30,31].

The involvement of NOS in QUIN toxicity on human astrocytes and neurons has been demonstrated [7,32,33]. This neurotoxic involvement of NOS has been confirmed by the use of the NOS inhibitor, nitro-L-arginine methyl ester, which can protect human primary neurons and astrocytes *in vitro* against QUIN toxicity [7,34]. NOS inhibitors have also been found to be effective in protecting mice and monkey models from the development of AD pathophysiology [35].

Another way to attenuate increased  $\text{NO}\bullet$  production and consequent energy depletion due to QUIN is to block the NMDA receptor. We have previously shown that the NMDA ion channel blocker, MK-801, can protect human neurons from QUIN-induced excitotoxicity [7]. However, long-term NMDA receptor inhibition by MK-801 has previously been shown to be toxic to cultures of rat cortical neurons [36]. Alternatively, polyphenols with their ROS/RNS scavenging, metal chelating and anti-inflammatory properties represent a promising additional option for the modulation of excitotoxic cell death that may potentially be effective in conditions such as AD treatment (Fig. 8). The neuroprotective effects of green tea polyphenols and their potential in the treatment of AD have been extensively reviewed [19,37,38].

In this study, we evaluated the effects of several polyphenolic compounds on QUIN-mediated elevations in nNOS activity and nitrite production. The activity of nNOS was considerably enhanced in a dose-dependent manner, with increasing concentrations of QUIN within 30 min, with a subsequent increase in nitrite production (Fig. 1). These results are consistent with previous reports showing increased  $\text{NO}\bullet$  production in the striatum within 2 h of QUIN injection [32,33].

Conversely, a dose-dependent decrease in nNOS activity and nitrite production was observed in QUIN-treated neuronal cells preincubated with selected polyphenolic compounds (Fig. 1). EPCG, catechin hydrate and curcumin showed a greater inhibitory effect on nNOS activity and subsequent nitrite production compared with apigenin, naringenin and gallotannin (Fig. 1). The modulatory effect of polyphenolic compounds on the NOS family has been previously reviewed in [19]. EPCG, catechin hydrate and curcumin can suppress  $\text{NO}\bullet$  production in cultures of RAW 264.7 macrophages and human peripheral blood mononuclear cells following a 24 h stimulation with lipopolysaccharide [39]. Moreover, apigenin has been shown to downregulate iNOS expression and  $\text{NO}\bullet$  production in RAW 264.7 macrophages [40]. Taken together, these results suggest that polyphenols can

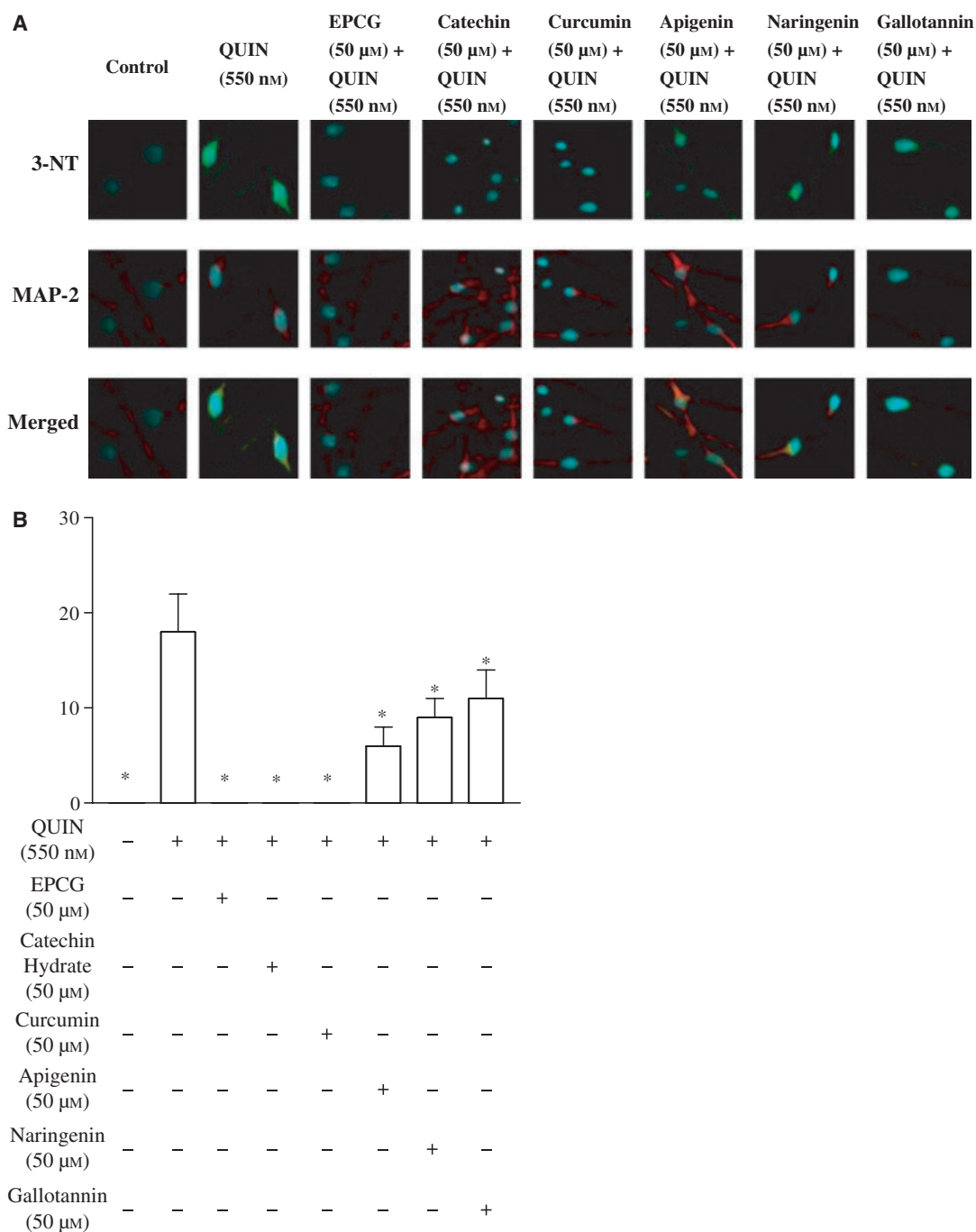


**Fig. 5.** Effect of polyphenols on QUIN-induced  $\text{Ca}^{2+}$  influx in human neurons. Representative trace of intracellular  $\text{Ca}^{2+}$  induced by 550 nM QUIN in the presence of: (A) EPCG, (B) catechin hydrate, (C) curcumin, (D) apigenin, (E) naringenin, (F) gallotannin. (G) Quantified amplitude of neuronal response to QUIN and EPCG, catechin hydrate, curcumin, apigenin, naringenin and gallotannin. The polyphenols were washed out during QUIN administration, as the polyphenols may influence its fluorescence ( $*P < 0.05$  compared with 550 nM QUIN;  $n = 4$  for each treatment group).

inhibit  $\text{NO}\bullet$  production by significantly reducing iNOS expression and activity. However, the present study was the first to examine the inhibitory effects of polyphenolic compounds on nNOS activity in primary cultures of human neurons. Consistent with the above results, EPCG, catechin hydrate and curcumin showed a significant reduction in 3-NT formation compared with QUIN-treated cells alone (Fig. 6). Apigenin, naringenin and gallotannin also exerted a protective effect against 3-NT formation, but to a lesser extent than the other polyphenols (Fig. 6).

We have previously shown that QUIN can induce PARP-1 activity and subsequent  $\text{NAD}^+$  depletion in primary cultures of human astrocytes and neurons at pathophysiological concentrations [7]. In that earlier

study, NOS inhibition using nitro-L-arginine methyl ester significantly reduced  $\text{NAD}^+$  depletion and PARP-1 activation in cultured human neurons exposed to cytotoxic concentrations of QUIN [7]. The present study showed that the polyphenols, EPCG, catechin hydrate and curcumin, which have a greater inhibitory effect on nNOS activity and nitrite production, can prevent DNA damage [indicated by reduced PAR formation (Fig. 7) and PARP-1 activation (Fig. 3)] and block the subsequent depletion of  $\text{NAD}^+$  stores, thereby preserving the cell's energy-dependent functions (Fig. 3). Apigenin, naringenin and gallotannin also showed a neuroprotective effect against PARP-1 activation and  $\text{NAD}^+$  depletion, but to a lesser extent than the previously mentioned polyphenols, probably

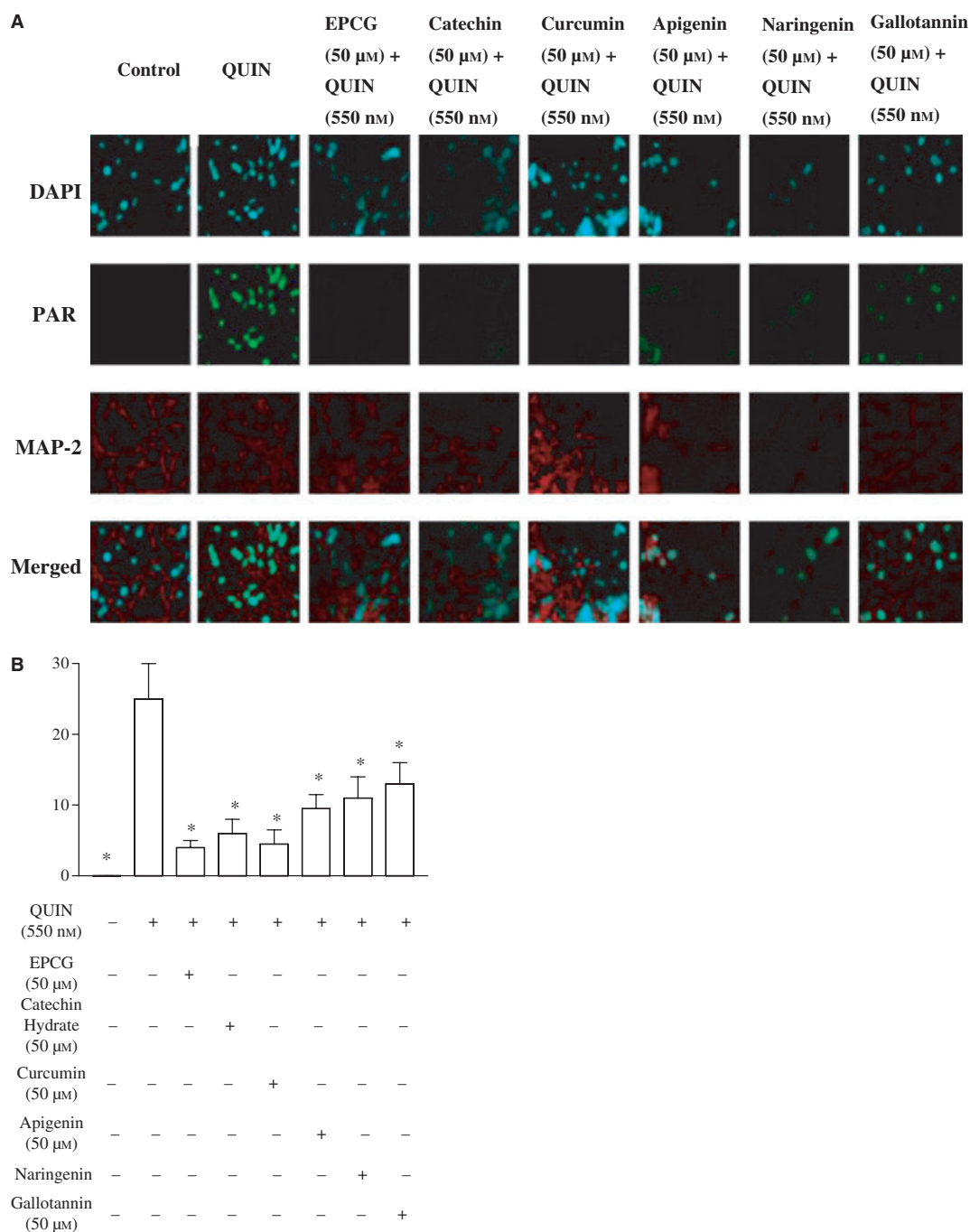


**Fig. 6.** Immunocytochemical detection of 3-NT in purified primary human neurons after QUIN (550 nM) stimulation. Staining for 3-NT in human neurons: top row – double staining for 3-NT/green and DAPI/blue; centre – double staining for MAP-2/red and DAPI/blue; bottom row – merged 3-NT/green, MAP-2/red and DAPI/blue. (B) Numeration of fluorescence intensity of 3-NT in human neurons using immunocytochemistry. The histogram shows the percentage of human neurons expressing 3-NT relative to the total number of neuronal cells after 24 h of treatment ( $*P < 0.05$  compared with 550 nM QUIN alone);  $n = 4$  for each treatment group.

due to their lower inhibitory effect on nNOS activity (Fig. 3).

Although treatment with catechin hydrate, apigenin and naringenin alone showed no significant difference

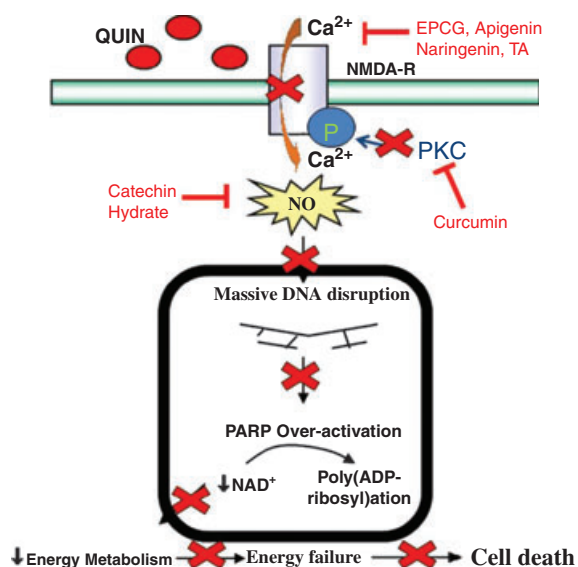
in intracellular  $\text{NAD}^+$  levels, and PARP and LDH activities across the range of concentrations tested, increased intracellular  $\text{NAD}^+$  levels were observed following treatment with EPCG and curcumin alone



**Fig. 7.** Immunocytochemical detection of PAR in purified primary human neurons after QUIN (550 nM) stimulation. Staining for PAR in human neurons: top row – nuclear staining for DAPI/blue; second row – staining for PAR/green; third row – double staining for DAPI/blue and MAP-2/red; fourth row – merged PAR/green, MAP-2/red and DAPI/blue. (B) Numeration of fluorescence intensity of PAR in human neurons using immunocytochemistry. The histogram shows the percentage of human neurons expressing PAR relative to the total number of neuronal cells after 1 h of treatment (\* $P < 0.05$  compared with 550 nM QUIN alone);  $n = 4$  for each treatment group.

(Fig. 2). This is consistent with the observation that PARP activity (and therefore  $\text{NAD}^+$  turnover) was also lowest following treatment with both EPCG and curcumin at 50 and 100  $\mu\text{M}$  (Fig. 2B). On the other

hand, gallotannin showed a dose-dependent decrease in intracellular  $\text{NAD}^+$  levels (Fig. 2A), with a corresponding decrease in cell viability (Fig. 2C). This may be explained by the observation by others that gallo-



**Fig. 8.** Schematic representation of the protective effects of EPCG, curcumin, catechin hydrate, apigenin, naringenin and gallotannin. The excitatory neurotoxin QUIN leads to over-activation of NMDA receptors followed by sustained Ca<sup>2+</sup> influx. The Ca<sup>2+</sup> influx leads to the formation of NO• by the activation of nNOS. Highly reactive free radicals are formed, which can cause oxidative damage to DNA leading to over-activation of PARP-1 and subsequent NAD<sup>+</sup> depletion and cell death due to energy restriction. Polyphenols can inhibit QUIN-induced excitotoxicity. However, each polyphenolic compound exerts its neuroprotective effect through a distinct mechanism.

tannin strongly inhibits nuclear nicotinamide mononucleotide adenylyltransferase (NMNAT-1) activity, with no detectable activity observed at 100  $\mu\text{M}$  [41].

The results of the present study show that QUIN can induce intracellular Ca<sup>2+</sup> influx in a dose-dependent manner (Fig. 4), and that this reduces the viability of cultured human neurons. To determine whether the neuroprotective effect of these polyphenols was due to a direct nNOS inhibition or via intracellular Ca<sup>2+</sup> modulation, we examined the effect of these polyphenols on intracellular Ca<sup>2+</sup> influx in human neurons following QUIN stimulation. We found that EPCG and curcumin were able to attenuate QUIN-induced Ca<sup>2+</sup> influx to a greater extent than apigenin, naringenin and gallotannin (Fig. 5). However, catechin hydrate did not attenuate the observed increase in Ca<sup>2+</sup> in QUIN-treated neuronal cultures (Fig. 5). EPCG has been previously shown to attenuate glutamate-induced cytotoxicity via intracellular ionotropic Ca<sup>2+</sup> modulation in PC12 cells, although the exact mechanism remains unclear [42]. Curcumin has been shown to exert a potent antioxidant effect on NO•-related radical generation [43]. Curcumin has also been shown to antagonize several important pathways

involved in NOS-mediated neurotoxicity, including activation of nuclear factor kappa B, the Jun N-terminal kinase pathway and protein kinase C [26,44,45]. Protein kinase C partly phosphorylates the core NMDA receptor subunit NR1, which potentiates increased Ca<sup>2+</sup> influx following NMDA receptor activation [26]. A decreased phosphorylation of NR1 may protect against QUIN-induced excitotoxicity when the levels of QUIN are significantly elevated. We found that catechin hydrate did not reduce QUIN-induced Ca<sup>2+</sup> influx in human neurons. This is consistent with another study, where catechin hydrate only slightly inhibited the phosphorylation of protein kinase C [26]. However, catechin hydrate significantly reduced QUIN-induced nNOS activity and NO• production. It is possible that inhibition of nNOS activity by catechin hydrate may be mediated through a direct action on the enzyme itself. For example, nitrite and peroxy-nitrite inhibition by catechins has been attributed to the 3'4'-catechol group on the B-ring [26].

Apigenin and naringenin are known to protect against excitotoxic insults in human neurons independent of NOS activity. Silva *et al.* [46] showed that the apigenin derivative biapigenin prevented kainate excitotoxicity by protecting cultured neurons from delayed Ca<sup>2+</sup> deregulation due to excessive NMDA receptor activation. Further studies have focussed on the binding of naringenin to GABA<sub>A</sub> receptors as a potential neuroprotective mechanism of action in the central nervous system [47,48].

Our results show that gallotannin is less active against nNOS activity and demonstrated poor nitrite scavenging properties (Fig. 1). However, gallotannin was able to attenuate QUIN-induced Ca<sup>2+</sup> influx in human primary neurons to a similar extent as apigenin. Other studies have shown that gallotannin can only significantly reduce Ca<sup>2+</sup> influx when administered simultaneously with glutamate [26]. This suggests a possible competitive inhibitory process.

Importantly the concentrations used in these experiments are within the achievable range of serum levels following oral consumption of these polyphenols. For example, one human study reported that the serum concentration of curcumin was  $1.77 \pm 1.87 \mu\text{M}$  [49]. In another rat study, daily oral consumption of a glyconated form of catechin resulted in a serum concentration of  $34.8 \pm 6.0 \mu\text{M}$  [50]. The amount of EPCG in a single cup of green tea is  $\sim 300 \mu\text{M}$  [51]. Therefore, the calculated maximum serum concentration of EPCG may reach 60  $\mu\text{M}$  in a 60 kg human after oral consumption of a single cup of tea. In the present study, the polyphenols were tested at a standardized concentration of 50  $\mu\text{M}$ . Although this concentration is rele-

vant to serum levels in humans, lower concentrations of these polyphenols may also be neuroprotective if administered over a longer period of time.

Several epidemiological studies have predicted neurodegenerative diseases to be a major public health problem in the 21st century [52]. In Australia it has been projected that although the total aging population will increase by 40% in 2042, the population with AD will increase by 3.5 times due to aging population demographics [53]. The neuroprotective effects of these green tea polyphenols were obtained in an experimental pretreatment model. The efficacy of these polyphenols *in vivo* is dependent on the ability of these polyphenols to cross the blood–brain barrier. Curcumin, EPCG and catechin have been reported to pass through the blood–brain barrier [54,55]. The permeability of apigenin, naringenin and gallocatechin remains unknown.

In a recent meta-analysis of 187 retrospective studies, EPCG, curcumin, catechin hydrate, melatonin, resveratrol, vitamin C and vitamin E were identified as naturally occurring compounds that show efficiency in slowing down the spectre of AD symptoms [56]. The results from our study and others add support to this observation and may encourage individuals to select foods that contain these beneficial compounds (e.g. red grapes, blue berries, peanuts, etc.). This will be important to improve population health in general, and in aging populations in particular.

## Materials and methods

### Reagents and chemicals

Dulbecco's phosphate buffer solution, Fura-2-AM fluorophore and all other cell culture media and supplements were obtained from Invitrogen (Melbourne, Australia) unless otherwise stated. Nicotinamide, bicine,  $\beta$ -NADH, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide, Hepes, D-glucose, alcohol dehydrogenase, sodium pyruvate, Tris,  $\gamma$ -globulins, QUIN, 4',6-diamidino-2-phenylindole dihydrochloride (DAPI), EPCG, catechin hydrate, curcumin, apigenin, naringenin and gallocatechin were obtained from Sigma-Aldrich (Castle-Hill, Australia). Phenazine methosulfate was obtained from ICN Biochemicals (Aurora, OH, USA). Bradford reagent was obtained from BioRad (Hercules, CA, USA). Rabbit anti-microtubule-associated protein 2 (MAP2) was obtained from Millipore (Melbourne, Australia). Mouse anti-poly(ADP-ribose) (10H) was obtained from Alexis Corporation (Pastlach, Switzerland). Mouse anti-3-NT, secondary anti-mouse IgG and anti-rabbit Alexa 488 (green)- or Alexa 594 (red)-conjugated IgG were obtained from Molecular Probes (Eugene, OR, USA). All commercial

antibodies were used at the concentrations specified by the manufacturer.

### Cell cultures

Human fetal brains were obtained from 16–19-week-old fetuses collected following therapeutic termination with informed consent. Mixed brain cultures were prepared and maintained using a protocol previously described by Guillemain *et al.* [2]. Neurons were prepared from the same mixed brain cell cultures as previously described [29]. Briefly, cells were plated in 24-well culture plates coated with Matrigel (1/20 in Neurobasal) and maintained in Neurobasal medium supplemented with 1% B-27 supplement, 1% Glutamax, 1% antibiotic/antifungal, 0.5% Hepes buffer and 0.5% glucose. The cells were maintained at 37 °C in a humidified atmosphere containing 95% air/5% CO<sub>2</sub>.

### Measurement of nNOS activity using the citrulline assay

nNOS activity was assayed by monitoring the conversion of L-[<sup>3</sup>H]arginine to L-[<sup>3</sup>H]citrulline, as previously described [57]. The cells were treated with 50–1200 nM QUIN for 30 min. After incubation, the reaction was terminated by adding 0.3 M HClO<sub>4</sub> (pH 5.5) containing EDTA (4 mM). Radiolabelled citrulline is neutral at a pH of 5.5, and was separated from the positively charged arginine using a column containing analytical grade cation-exchange resin (AG Dowex 50W-X8). The amount of L-[<sup>3</sup>H]citrulline was measured using a Beckman LS6500 scintillation counter. The results were expressed as ng L-citrulline/500  $\mu$ g protein<sup>-1</sup>·30 min<sup>-1</sup>. In another set of experiments, neuronal cells were preincubated for 15 min with 1–100  $\mu$ M EPCG, catechin hydrate, curcumin, apigenin, naringenin and gallocatechin. The nNOS activity in the presence of 550 nM QUIN was then quantified as described above.

### Nitrite determination by fluorometric Griess diazotization assay

Nitrite production in the culture supernatant was measured using the fluorometric Griess diazotization assay, as previously described [57]. In the Griess assay, NO<sub>2</sub> is allowed to react with an aromatic amine in acidic medium to yield a fluorescent azo derivative. Briefly, neurons were treated with 50–1200 nM QUIN for 30 min and 100  $\mu$ L culture supernatant was placed in a 96-well microplate. Diaminonaphthalene was diluted to 10 mM in deionized water from the original 100 mM dimethylsulfoxide stock solution, and 1% HCl was added to the aqueous mixture to generate a working stock of diaminonaphthalene. Then, 100  $\mu$ L diaminonaphthalene was added to each sample and incubated for 10 min at room temperature. An additional 100  $\mu$ L 2 M

NaOH was added and the fluorescence intensity was then recorded at an excitation wavelength of 355 nm and an emission wavelength of 460 nm. In another set of experiments, neuronal cells were preincubated for 15 min with 1–100  $\mu\text{M}$  EPCG, catechin hydrate, curcumin, apigenin, naringenin and gallotannin. The amount of nitrite produced in the presence of 550 nm QUIN was then quantified as described above.

### Calcium influx studies using fluorometry

To measure intracellular  $\text{Ca}^{2+}$ , human neurons were loaded ( $\sim 1$  h, room temperature) with 3.5  $\mu\text{g}\cdot\text{mL}^{-1}$  Fura-2-AM in a loading solution containing (in mM): 135 NaCl, 5 KCl, 1  $\text{MgCl}_2$ , 1  $\text{CaCl}_2$ , 5 glucose and 10 Hepes (pH 7.4). Probenicid dissolved in 1 M NaOH was added to the loading solution at a final concentration of 4 mM to reduce dye leakage. Following the recommended 1 h incubation period, the loading solution was removed and replaced with 1x Hanks balanced salt solution (HBSS) containing 50 mM glycine. The addition of selected polyphenols (EPCG, catechin hydrate, curcumin, apigenin, naringenin and gallotannin) was undertaken 15 min before the addition of QUIN to ensure that adequate diffusion time was provided to attain equilibrium. The  $\text{Ca}^{2+}$  influx experiments were subsequently performed using a Fluostar Optima fluorometer (Durham, NC, USA). Filter excitation and emission was set at 485 and 520 nm wavelengths, respectively. For each well, fluorescence was measured via orbital scanning of 10 locations at a 3 mm radius every 0.5 s, and the average of these readings was recorded. Baseline fluorescence was measured during the first 10 s of the experiment, followed by injection of QUIN (in HBSS). Fluorescent readings were subsequently taken for an additional 90 s. Negative controls included injection of only HBSS solution without an agonist.

### NAD(H) microcycling assay for the measurement of intracellular $\text{NAD}^+$ concentrations

The intracellular  $\text{NAD}^+$  concentration was measured spectrophotometrically using the thiazolyl blue microcycling assay established by Bernofsky & Swan [58] adapted for the 96-well plate format by Grant & Kapoor [59]. Human neurons were preincubated for 15 min with 30 and 50  $\mu\text{M}$  EPCG, catechin hydrate, curcumin, apigenin, naringenin and gallotannin. The cells were then treated with QUIN (550 nm) and intracellular  $\text{NAD}^+$  levels were measured 24 h later.

### Extracellular LDH activity as a measurement for cytotoxicity

The release of LDH into culture supernatant correlates with the amount of cell death and membrane damage, providing

an accurate measure of cellular toxicity. LDH activity was assayed using a standard spectrophotometric technique described by Koh & Choi [60]. After preincubation with 1–100  $\mu\text{M}$  EPCG, catechin hydrate, curcumin, apigenin, naringenin and gallotannin, neuronal cells were treated with QUIN (550 nm) and extracellular LDH activity was assessed in culture supernatant after 24 h.

### PARP assay for the measurement of intracellular PARP activity

PARP activity was measured using a new operational protocol relying on the chemical quantification of  $\text{NAD}^+$  modified from Putt *et al.* [61] and adapted for the 24-well format [7]. After a 15 min preincubation with the selected polyphenolic compounds, neurons were treated with QUIN (550 nm) and incubated for 15 min. Dulbecco's phosphate buffer solution was then aspirated and PARP lysing buffer (200  $\mu\text{L}$ ) was added to the cell plate. The buffer solution contained  $\text{MgCl}_2$  (10 mM), Triton X-100 (1%) and  $\text{NAD}^+$  (20  $\mu\text{M}$ ) in Tris buffer (50 mM, pH 8.1). The plate was then incubated for 1 h and PARP activity was assayed as previously described [7].

### Bradford protein assay for the quantification of total protein

$\text{NAD}^+$  concentration, PARP and extracellular LDH activities were adjusted for variations in cell number using the Bradford protein assay [62].

### Immunocytochemistry for the detection of PAR and 3-NT formations

The method for immunocytochemistry has been previously described [2]. Cells were incubated with mAb PAR and mAb 3-NT together with the phenotypic marker (MAP-2). Selected secondary antibodies (goat anti-mouse IgG or goat anti-rabbit coupled with Alexa 488 or Alexa 594) were used. The following controls were performed for each labelled experiment: (a) isotypic antibody controls and (b) incubation with only the secondary labelled antibody. Cell counting was performed in a blind manner. The whole controls and untreated chamber slides were counted. Enumeration of each slide was classified according to the following scheme: DAPI staining for total cell number, MAP-2 immunoreactivity for neurons, and 3-NT and PAR staining.

### Data analysis

The results obtained are presented as the means  $\pm$  standard error of measurement. One-way analysis of variance and posthoc Tukey's multiple comparison tests were used

to determine the statistical significance between treatment groups. Differences between treatment groups were considered significant if  $P < 0.05$ .

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