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Prevention of neuronal cell damage induced by oxidative stress in-vitro: effect of different *Ginkgo biloba* extracts

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Abstract

The effect of two different *Ginkgo biloba* extracts (GB1 and GB4) was studied in-vitro on cultured neurons exposed to oxidative stress caused by H_2O_2(50 μmol L^{-1}) and $FeSO_4$(100 μmol L^{-1}). Only about 50% of the neurons were still viable at the end of the experiment (8 h) in control conditions, while the two extracts dose dependently increased the number of viable cells, in the concentration range 10–200 μg mL^{-1}. The two *Ginkgo biloba* extracts differed in their effect on hydroxyl-radical-scavenging capacity: GB1 and GB4 had an IC50 (50% inhibiting concentration) value of 78 μg mL^{-1} and 186 μg mL^{-1}, respectively. However, both extracts inhibited apoptosis in cortical neurons after oxidative stress in-vitro. These observations make one suppose that different preparations of *Ginkgo biloba* have quantitatively different actions and outline the importance of the contribution of apoptosis prevention toward their neuroprotective action.

Introduction

Oxidative stress has been recently considered as a key factor in various acute and chronic neurodegenerative diseases characterised by extensive cell death. It is well known that neuronal cell death can occur by either apoptosis or necrosis. Necrosis involves generalised breakdown of cellular structure and function followed by cell lysis and tissue inflammation, while apoptosis is characterised by cell shrinkage, membrane blebbing, formation of apoptotic bodies and fragmentation of nuclear DNA. Different oxidative cytotoxic agents induce apoptosis in different types of cell, including cortical neurons (e.g. H_2O_2, typically considered as a cause of oxidative stress), while antioxidants such as vitamin E or *N*-acetylcysteine inhibit cell apoptosis (Forrest et al 1994; Preston et al 1994; Whittemore et al 1994).

To reduce or prevent the extent of neuronal damage in the presence of oxidative stress, a number of different compounds and plant extracts have been studied. In particular, a *Ginkgo biloba* extract, EGb761, has been reported to protect the brain against hypoxic damage and to inhibit the formation of reactive oxygen species in rat brain neurons (Pincemail et al 1989; Oyama et al 1992). The same extract has been studied with positive results in double-blind controlled clinical trials in aged subjects with memory impairment due to moderate primary degenerative dementia of Alzheimer type or multi-infarct dementia (Kanowski et al 1997; Le-Bars et al 1997; Maurer et al 1997).

Extracts of *Ginkgo biloba* may vary in their chemical composition because of the

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different procedures used in their extraction. At present, however, it is not possible to attribute a given pharmacological activity to a precise panel of compounds present in a particular extract. Thus, a biological assay related to a specific pharmacological activity would be an essential addition to a good chemical characterisation by which different *Ginkgo biloba* preparations could be fully compared. On the basis of the available evidence, in-vitro experiments on neuronal cultures seem particularly useful to evaluate the possible neuroprotective action of *Ginkgo biloba* extracts.

Recent data on the action of two preparations emphasise this opportunity. A Chinese group, using EGb761 on rat cerebellar neurons, found that the extract protected against apoptotic death induced by hydroxyl radicals (Ni et al 1996), while in a similar in-vitro model a Japanese group showed protection against oxidative damage caused by H_2O_2 (Oyama et al 1996). Both groups studied the protective effect of *Ginkgo biloba* using only a single concentration, namely 100 μg mL^{-1} for the Chinese group and 10 μg mL^{-1} for the Japanese group. The different concentrations could have been because of differences in experimental conditions, but may also indicate a major difference in the eteroside and terpenoid contents of the extracts.

To examine this interesting topic, we performed a series of experiments using cultured neurons exposed to oxidative stress in the presence of various concentrations of two different *Ginkgo biloba* preparations, characterised by their content of flavone glycosides, terpene lactones and proanthocyanidins, using acetylcysteine as a reference compound. The mechanism of the neuroprotective action was characterised by measuring the hydroxyl-free-radical-scavenging properties and the extent of cell apoptosis, confirming that *Ginkgo biloba* extracts were able to prevent apoptosis induced by free oxygen radicals.

Materials and Methods

Materials

Deoxyribonuclease, ethidium bromide, poli-D-lysine, RNAse A and trypsin were purchased from Sigma Aldrich S.r.l.; agarose, Eagle's minimum essential medium, fetal calf serum and proteinase K were products of Gibco BRL.

Description of extracts

Ginkgo biloba extract GB1 was manufactured by Linnea. Briefly, the milled leaves of *Ginkgo biloba* had been extracted with aqueous acetone and the resulting extract, after treatment with different organic solvents, was purified, concentrated and dried. Analysis showed the following content: flavone glycosides 26.8%; terpene lactones 6.51%; proanthocyanidins 4.74%; and ginkgolic acids 0.17 ppm; microbiological purity complied with Ph. Eur. specifications.

Extract GB4 was obtained from Indena, and was representative of extracts manufactured by leading companies in the field of extraction of vegetable active ingredients. Analysis of GB4 yielded: flavone glycosides 24.89%; terpene lactones 6.11%; proanthocyanidins 3.38%; and ginkgolic acids 0.34 ppm; microbiological purity complied with Ph. Eur. specifications.

Cell culture and viability estimation

Cortical neurons were obtained from brain tissue removed from fetal rats at embryonic day 17. Cortical cells were dissociated in serum-free medium containing 0.1% trypsin and 25 mg mL^{-1} deoxyribonuclease for 5 min at room temperature and plated in 24-well dishes, pre-coated with poly-D-lysine (50 mg mL^{-1}). The cells were cultured in Eagle's minimum essential medium supplemented with 10% fetal calf serum and glutamine (2 mmol L^{-1}). Cultures were kept at 37°C in humidified CO_2 atmosphere.

After 3–5 days, in-vitro non-neuronal cell division was halted by exposure to cytosine arabinoside, an inhibitor of mitosis, to prevent overgrowth of glial cells.

After 3 more days, neurons were exposed to 50 μmol L^{-1} H_2O_2 + 100 μmol L^{-1} $FeSO_4$ and incubated for 8 h. The control cells were incubated under the same conditions without H_2O_2 and ferrous sulphate. Neurons were treated with *Ginkgo biloba* extracts (10, 50, 100 and 200 μg mL^{-1}) 60 min before oxidative-stress induction.

In a separate series of experiments, *Ginkgo biloba* extracts and acetylcysteine were tested in the absence of oxidative stress to verify the absence of intrinsic neuronal toxicity. Cell viability was quantitatively assessed by crystal violet staining (0.5% in water–methanol, 4:1). After washing, cells were dried and densitometric analysis of staining was carried out using an image analyser (Cafè et al 1996). Results were expressed as the percentage of viable cells, calculated considering 100% survival to be the values observed in the absence of test substances and of oxidative stress. Each value was the mean of 30 replications obtained in 5 different experiments and the significance of the difference was calculated using Student's *t*-test.

Scavenging of hydroxyl radicals

Deoxyribose degradation caused by hydroxyl radicals was quantified by measuring the formation of a chromogenic substance obtained by reacting thiobarbituric acid with sugar degradation products such as malonaldehyde (Halliwell et al 1987) at 532 nm. Hydroxyl radicals were generated by mixing ascorbic acid (100 μmol L^{-1}), H_2O_2 (5 μmol L^{-1}) and ferric chloride (10 μmol L^{-1}).

Ferric chloride reacts with H_2O_2 to generate hydroxyl radicals that attack deoxyribose. The rate of deoxyribose degradation may be increased by including a reducing agent, such as ascorbic acid. The reaction mixture, with GB1 and GB4 (and without extracts in the control sample), were incubated at 37°C for 1 hour and colour developed.

Apoptosis

DNA fragmentation, measured as an index of apoptosis, was determined after cell lysis followed by harvesting and centrifugation using agarose gel electrophoresis of cellular lysates (Forloni et al 1993).

Cells (10^6) were centrifuged at 2000 rev min^{-1} for 3 min and lysed in lysis buffer (10 mmol L^{-1} Tris, 20 mmol L^{-1} 0.5% Triton X 100) at 4°C. The lysates were centrifuged at 13000 *g* to separate surpernatant and pellet.

The pellet was treated with another lysis buffer (50 mmol L^{-1} Tris, 10 mmol L^{-1} EDTA, 0.5% Sarcosil). The supernatant was treated with proteinase K (20 mg mL^{-1}) and, after an incubation of 12 h, with RNAse A (10 mg mL^{-1}). Pellet and supernatant were extracted with phenol to separate organic phase from aqueous phase. The aqueous phase was treated with phenol–chloroform, 1:1, and then with chloroform to extract DNA. DNA was precipitated with isopropyl alcohol and NaCl 5 mol L^{-1} at −70°C for 12 h. After two centrifugations at 13000 *g*, samples were kept at 37°C for 12 h.

DNA coloured with ethidium bromide was charged on agarose gel (1.0–1.2%) and electrophoresed at 65 V.

Results

The content of flavone glycosides and terpene lactones was quite similar in the two *Ginkgo biloba* samples, 25–27% and 6.1–6.5% in GB1 and GB4, respectively. The content of proanthocyanidins was 4.7 and 3.4 % in GB1 and GB4, respectively.

The two different *Ginkgo biloba* extracts and the reference compound, acetylcysteine, were initially tested at concentrations of 10, 50, 100 and 200 μg mL^{-1} in the absence of free radicals, to verify the absence of direct cellular toxicity of the extracts (data not shown). While the experimental preparations were free of toxicity at concentrations of up to 200 μg mL^{-1}, acetylcysteine (at 200 μg mL^{-1}) caused partial cell death. Thus, in the following experiments the highest tested concentration was 100 μg mL^{-1}.

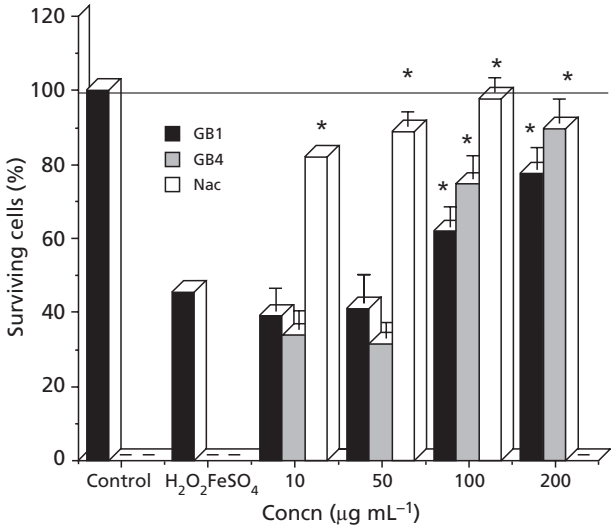

Figure 1 Effect of *Ginkgo biloba* extracts (GB1 and GB4) and of acetylcysteine (Nac) on rat fetal cortical neuron cell survival (% of total) after oxidative stress induced by the presence of H_2O_2 (50 μmol L^{-1}) and $FeSO_4$ (100 μmol L^{-1}). The extracts and the reference compound were added to cultured cells 60 min before stress induction and survival was evaluated after 8 h. $*P < 0.01$, compared with cells exposed to oxidative stress in the absence of drugs.

Oxidative stress in our experimental condition resulted in about 50% of cell death, as indicated by a clear decrease in the uptake of the vital dye, crystal violet. The *Ginkgo biloba* extracts reduced neuronal cell death dose dependently (Figure 1). The maximal protective activity of the two extracts was similar, with cell survival increasing to about 80%. Acetylcysteine was also active, the effect being significant at all the tested concentrations (10, 50 and 100 μg mL^{-1}).

In another series of experiments we tested the capability of the extracts to act as hydroxyl-radical scavengers in-vitro, in an experimental system without living cells. Figure 2 demonstrates that the two tested preparations shared the capacity to act as radical scavengers in-vitro.

Finally, the action of the *Ginkgo biloba* extracts on apoptosis was analysed by measuring DNA fragmentation as a marker of apoptotic damage induced by free radicals. Figure 3 shows that oxidative stress caused

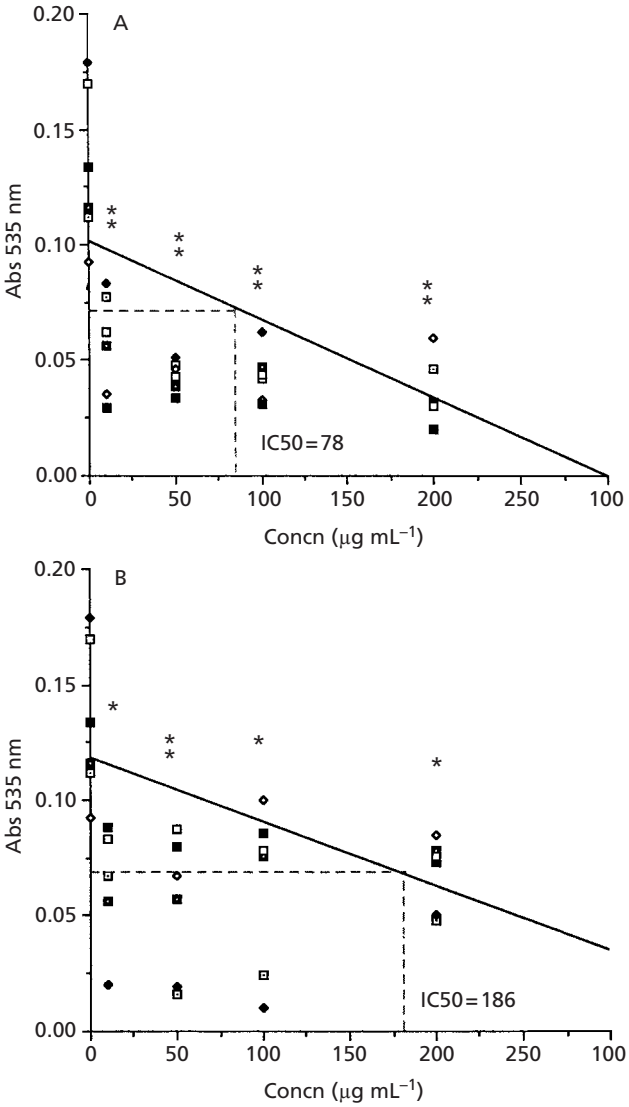

Figure 2 Absorption at 535 nm related to substances reacting with thiobarbituric acid (TBARs) obtained by the reaction between $OH^{\cdot}$ and deoxyribose, in the presence of GB1 (A) and GB4 (B), and calculation of IC50 values relevant to GB1 and GB4. Geometrical symbols represent different experimental points. $^{*}P < 0.05$; $^{**}P < 0.01$, compared with control.

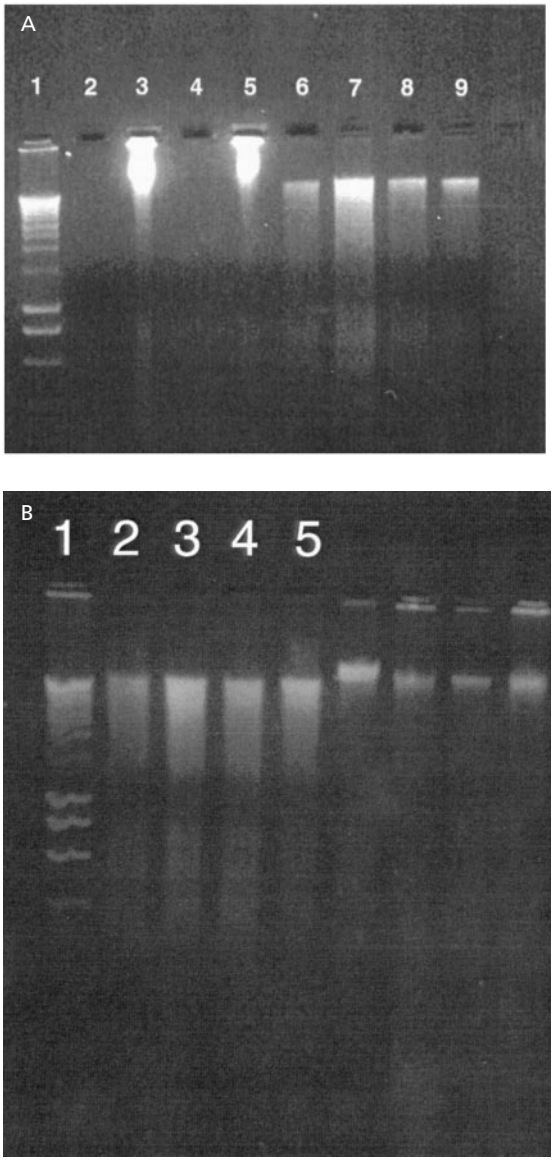

Figure 3 Effect of GB1 and GB4 on apoptosis of rat fetal cortical neuron cells. A. Track 1, reference fragmented DNA. Tracks 2–5, fragmentation of high-molecular-weight DNA. Track 6, control cortical cells with limited DNA fragmentation. Track 7, DNA fragmentation (index of apoptosis) of cells exposed to oxidative stress caused by the presence of H_2O_2 (50 μmol L^{-1}) and $FeSO_4$ (100 μmol L^{-1}). Track 8, cells treated only with GB1 (100 μg mL^{-1}). Track 9 indicates the neuroprotective effect exerted by GB1 (100 μg mL^{-1}) on cells exposed to oxidative stress; DNA fragmentation did not occur, and so there was no apoptosis. B. Track 1, reference fragmented DNA. Track 2, control cortical cells. Track 3, DNA fragmentation (index of apoptosis) of cells exposed to oxidative stress caused by the presence of H_2O_2 (50 μmol L^{-1}) and $FeSO_4$ (100 μmol L^{-1}). Track 4, cells treated only with GB4 (100 μg mL^{-1}). Track 5 indicates the neuroprotective effect exerted by GB4 (100 μg mL^{-1}) on cells exposed to oxidative stress.

massive fragmentation, as indicated by the presence of low-molecular-weight DNA fragments, whereas incubation with GB1 and GB4 reduced DNA fragmentation, thus positively influencing the apoptosis mechanisms.

Discussion

These results confirm that *Ginkgo biloba* extracts, GB1 and GB4, exert a protective effect on cultured neurons

in the presence of neurotoxic free radicals. Our observations also indicate that this interesting effect is shared by different preparations and is dose dependent. Furthermore, the protective effect can be observed by measuring either cell survival or apoptosis and is accompanied by potent activity as a scavenger of toxic free radicals, namely hydroxyl radicals. Our data generally give support to the hypothesis that *Ginkgo* extracts have a strong anti-apoptotic and neuroprotective activity mediated, at least in part, by their ability to scavenge toxic free radicals. For the first time, however, it has been observed by direct comparison in the same experimental setting that different preparations, namely GB1 and GB4, exert quantitatively different hydroxyl-radical-scavenging activities ($IC50 = 78\ \mu g\ mL^{-1}$ for GB1 and $186\ \mu g\ mL^{-1}$ for GB4). Both GB1 and GB4 preparations conform to the requirements of the German Commission E Monograph, but nevertheless, some quantitative differences exist among the known chemical constituents that could explain the difference in the scavenging capacity.

However, it must be considered that more than 50 % of the content of the extract has not been chemically characterised and it is quite possible that different preparative techniques result in different contents of unknown, possibly active, substances. The comparative neuroprotective actions of the *Ginkgo biloba* extracts and specific compounds such as ginkgolide B, ginkgolide J and bilobalide differ insofar as preventing apoptosis in cultured chick embryonic neurons as well as in mixed cultures of hippocampus neurons and astrocytes (Ahlemeyer et al 1999). *Ginkgo biloba* and several of its constituents possess anti-apoptotic capacity, bilobalide being the most potent studied constituent, thus confirming that in a complex system like an extract it is practically impossible to ascertain if one single substance is responsible for biological activity.

The total flavonoid component of EGb761, two pure EGb761 components (rutin and quercetin) and a mixture of flavonoids and terpenes protected cerebellar granule cells from oxidative damage and apoptosis induced by hydroxyl radicals (Chen et al 1999). The total terpenes of EGb761 did not protect against apoptosis. EGb761 and flavonoids were able to scavenge hydroxyl radicals, while terpenes did not scavenge hydroxyl radicals (Chen et al 1999).

Experimental observations suggest that scavenging activity is essential for anti-apoptotic action, but the relevance of different scavenging capability is difficult to correlate to biological and pharmacological properties. We suggest that in-vitro work with a sensitive assay may ascertain whether different methods of preparation give equivalent products, at least in terms of biological activity. At present it is not possible to attribute any difference to a specific component, but the biological method employed appears to be sensitive and reproducible and may be useful to further examine the problem.

From a more general point of view, our data clearly demonstrate that different *Ginkgo* preparations, namely GB1 and GB4, share a very interesting in-vitro pharmacological property, which consists of reducing neuronal apoptosis induced by toxic free radicals, illustrating that the study of their use in neurodegenerative diseases is worth pursuing.

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