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EFFECTS OF APOCYNIN AND ETHANOL ON INTRACEREBRAL HAEMORRHAGE-INDUCED BRAIN INJURY IN RATS

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SUMMARY

1. In the present study, we investigated whether the administration of apocynin, an NADPH oxidase inhibitor, provided brain protection in a rat model of intracerebral haemorrhage (ICH).

2. Rats were divided into sham, ICH untreated, ICH treated with vehicle (ethanol) and ICH treated with apocynin groups. Intracerebral haemorrhage was induced by collagenase injection. Neurological function, haemorrhage volume and brain oedema were measured 24 h after ICH.

3. Intracerebral haemorrhage caused significant neurological deficit associated with brain oedema. Apocynin (3, 10 and 30 mg/kg) failed to reduce brain injury after ICH. Low dose ethanol (0.2 g/kg) improved neurological function and reduced brain oedema (ICH-vehicle *vs* ICH-untreated, $P < 0.05$).

4. In conclusion, apocynin has no neuroprotective effect when administered intraperitoneally after ICH.

Key words: apocynin, collagenase, ethanol, intracerebral haemorrhage, oedema, oxidative stress.

INTRODUCTION

Intracerebral haemorrhage (ICH) is a common and often fatal type of stroke in which the 30 day mortality rate is two- to sixfold higher than that for ischaemic stroke.[1] An effective therapy for ICH-induced secondary brain injury is currently not available.[2] Oxidative stress largely contributes to acute brain injury after ICH.[3,4] Although several interventions, including hypothermia, iron chelation and matrix metalloproteinase inhibitory agents, reduce oxidative damage after experimental ICH, treatment specifically targeting mechanisms of oxidative stress is still awaited.[5–7]

In addition to peroxisomes and the respiratory chain, NADPH oxidase is a major producer of superoxides in the brain.[8] Although a substantial source of brain NADPH activity seems to be the cerebral vasculature,[9] a multitude of studies has shown a strong expression of NADPH oxidase subunits in the neurons and glia of the central nervous system (CNS).[10–12] Cerebral ischaemia induces a robust increase in brain NADPH oxidase expression/activity, as does subarachnoid haemorrhage.[11,13,14] A recent study demonstrated that the expression of the gp91^{phox} catalytic subunit of NADPH oxidase increased in the brain after ICH, resulting in enhanced lipid peroxidation.[15] The deficiency of NADPH oxidase in gp91^{phox} knockout-mice reduced haemorrhage volume, brain oedema and neurological deficit after ICH.[15] Therefore, we hypothesize that pharmacological inhibition of NADPH oxidase will reduce oxidative stress and brain oedema and improve neurological function after ICH in rats.

Apocynin is a compound isolated from the medicinal plant *Picrorhiza kurroa*. Apocynin inhibits NADPH oxidase in neutrophils,[16,17] macrophages and systemic endothelia[18–20] through the inhibition of p47^{phox} subunit translocation. It has been reported that apocynin protects against global cerebral ischaemia–reperfusion-induced oxidative stress and injury in the gerbil hippocampus[21] and inhibiting superoxide production by NADPH oxidase with apocynin preserves blood–brain barrier constituents in ischaemia-like injury *in vitro*.[22] In the present study, we investigated whether apocynin was able to protect against brain injury in a collagenase-induced rat model of ICH.

METHODS

All the procedures for these studies were approved by the Animal Care and Use Committee at Loma Linda University and complied with the *Guide for the Care and Use of Laboratory Animals* (http://research.llu.edu/forms/appendixb.doc). Aseptic technique was used for surgery. Rats were allowed free access to food and water.

Experimental design

A total of 149 male Sprague-Dawley rats (300–380 g; Harlan, Indianapolis, IN, USA) was used in the present study. Rats were divided into sham, ICH untreated, ICH vehicle and ICH apocynin-treated groups. All animals were killed after neurological testing 24 h after ICH induction. Brain samples were collected to measure haemorrhage volume, brain oedema, lipid peroxidation and NADPH oxidase activity. The effects of ICH induction on blood pressure, blood gas and blood glucose levels were monitored in subsets of four rats.

Intracerebral haemorrhage induction

We adapted the collagenase-induced ICH model, as described previously in rats[23] and transgenic mice.[24] Briefly, rats were anaesthetized with ketamine (100 mg/kg, i.p.) and xylazine (5 mg/kg, i.p.) and positioned prone in a stereotaxic head frame (Kopf Instruments, Tujunga, CA, USA). An electronic thermostat-controlled warming blanket was used to maintain the core temperature at 37.0 ± 0.5°C. A cranial burr hole (1 mm) was drilled near the right coronal suture 2.9 mm lateral to the midline. A 27 gauge needle was inserted stereotaxically into the right basal ganglia (coordinates: 0.2 mm

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anterior, 5.6 mm ventral and 2.9 mm lateral to the bregma). Bacterial collagenase (VII-S; Sigma, St Louis, MO, USA; 0.2 U in 1 μL saline) in a Hamilton syringe was infused into the brain over 5 min at a rate of 0.2 μL/min with a microinfusion pump (Harvard Apparatus, Holliston, MA, USA). The needle was left in place for an additional 10 min after injection to prevent any possible leakage of the collagenase solution. After removal of the needle, the skull hole was closed with bone wax, the incision was sutured and rats were allowed to recover. Sham surgery was performed with needle insertion alone.

Physiological parameters

The right femoral artery was catheterized using PE-50 polyethylene tubing to monitor physiological parameters, including mean arterial blood pressure, blood gas and blood glucose levels (1610 pH/Blood Gas Analyser; Instrumentation Laboratories, Lexington, MA, USA). The physiological parameters were monitored before ICH induction, during collagenase injection and after surgery.

Apocynin treatment

Apocynin (Sigma-Aldrich, St Louis, MO, USA) was prepared in 100% ethanol at three concentrations (15, 50 and 100 mg/mL). Prior to injection, the apocynin–ethanol solution was diluted with saline to obtain doses of apocynin of 3, 10 and 30 mg/kg in 0.2 g/kg ethanol and the solution was injected intraperitoneally 2 h after ICH induction. Ethanol was used as the vehicle for apocynin after we failed to dissolve apocynin in saline or a low concentration of dimethylsulphoxide (< 1%). The effect of ethanol (0.2 g/kg) alone on ICH was tested in a separate animal group (ICH + Vehicle). In addition, the direct effect of ethanol on the activity of collagenase was tested in an *in vitro* study by zymography.

Neurological deficit

Neurological evaluation was conducted using an 18 point neurological scoring system[25] 24 h after ICH. The examiner had no knowledge of the procedure and the treatment that the rat had received. The neurobehavioural study consisted of six tests with scores of 1–3 for each test. These six tests included: (i) spontaneous activity; (ii) symmetry in the movement of four limbs; (iii) forepaw outstretching; (iv) climbing; (v) body proprioception; and (vi) response to vibrissae touch. The score given to each rat at the completion of the evaluation is the summation of all six individual test scores. The minimum neurological score is 3 and the maximum is 18.

Haemorrhage volume

A haemoglobin assay was conducted 24 h after ICH, as described previously.[24] Briefly, rats were killed with an overdose of isoflurane 24 h after ICH and perfused transcardially with 200 mL ice-cold phosphate-buffered saline (PBS). Brains were extracted and dissected free of olfactory bulbs and cerebellum. The ipsilateral hemisphere was homogenized (Tissue Miser Homogenizer; Fisher Scientific, Pittsburgh, PA, USA) for 60 s in a test tube containing distilled water (total volume 3 mL). After centrifugation (15 800 *g* for 30 min; model 5417R; Eppendorf, Hamburg, Germany), Drabkin's reagent (400 μL; Sigma-Aldrich) was added to 100 μL aliquots of the supernatant (four samples per brain) and allowed to react for 15 min. The absorbance of this solution was read using a spectrophotometer (540 nm; model Spectronic Genesis 5; Thermo Electron Corporation, Waltham, MA, USA) and the amount of blood in each brain was calculated using a curve generated previously using known blood volumes.

Brain water content

Brain water content was measured as described previously.[15,26] Briefly, rats were decapitated under deep anaesthesia. The brains were removed immediately and divided into three parts: ipsilateral hemisphere, contralateral hemisphere and cerebellum. The cerebellum was used as an internal control for brain water content. Tissue samples were weighed on an electronic analytical balance (APX-60; Denver Instrument, Denver, CO, USA) to the nearest 0.1 mg to obtain the wet weight (WW). The tissue was then dried at 100°C for 24 h to determine the dry weight (DW). Brain water content (%) was calculated as $(WW - DW)/WW \times 100$.

Lipid peroxidation

The level of lipid peroxidation products (malondialdehyde (MDA)) was measured using an LPO-586 kit (OxisResearch, Portland, OR, USA) in brain samples 24 h after ICH, as described previously.[15] Brains were homogenized in 20 mmol/L phosphate buffer (pH 7.4) and 0.5 mol/L butylated hydroxytoluene (BHT) in acetonitrile was added to prevent sample oxidation. Homogenates were centrifuged at 3000 *g* for 10 min at 4°C to remove large particles. Equal amounts of proteins in each sample were allowed to react with a chromogenic reagent at 45°C for 60 min. Samples were centrifuged at 15 000 *g* for 10 min at 4°C and supernatants were measured at 586 nm. The MDA level was calculated with a standard curve according to the manufacturer's instructions.

Activity of NADPH oxidase

The NADPH activity in brain tissues was measured as described previously.[11] Enzymatic activity was measured in whole hemispheres in order to avoid artefacts resulting from prolonged brain preparation.

Briefly, the ipsilateral hemispheres were collected 24 h after ICH and homogenized in Krebs'–Ringer phosphate buffer at pH 7.4 (120 mmol/L NaCl, 4.8 mmol/L KCl, 1.2 mmol/L $MgSO_4$, 2.2 mmol/L $CaCl_2$, 0.1 mol/L phospate buffer) with phenylmethylsulphonyl fluoride and a protease inhibitor cocktail (Sigma). Homogenates were centrifuged at 500 *g* for 5 min at 4°C and the pellet was discarded. The supernatants were spun at 100 000 *g* in a Beckman L5-5B ultracentrifuge (Beckman Coulter, Fullerton, CA, USA) for 1 h at 4°C. Cytosolic (supernatant) and membrane (pellet) fractions were then separated. The pellet was resuspended in 150 μL buffer and kept at –80°C until analysis. To assay NADPH oxidase enzymatic activity in the membrane fraction, 10 μL protein extract was added to each well of a Luminometer (Microlumat LB96P; Berthold Technologies, Oak Ridge, TN, USA) microplate, which contained 100 μL Krebs'–Ringer phosphate buffer supplemented with 100 μmol/L lucigenin (Molecular Probes, Eugene, OR, USA). The reaction was initiated by the addition of 10 μL NADPH solution (Sigma) to a final concentration of 100 μmol/L. Chemiluminescence counts were recorded every 90 s for 1 h. The respective background counts were subtracted and chemiluminescence was expressed in relative light units per μg protein.

Zymography

To test the direct effect of ethanol on collagenase, a collagenase–ethanol mixture was loaded on a 10% Tris–glycine gel with 0.1% gelatin as substrate (Bio-Rad, Hercules, CA, USA) and collagenase activities were determined by zymography as described previously.[24,27] Briefly, 1 μL of serial concentrations of collagenase (0.2, 0.02, 0.002, 0.0002 U) was mixed with 4 μL of 0.4% ethanol. The concentration of ethanol at 0.4% represents the concentration of ethanol in the brain of an ICH rat; the dose was estimated from 0.2 g/kg ethanol injected intraperitoneally that was then diluted by the systemic blood (approximately 15 mL) in the rat. The dose of collagenase used for inducing ICH was 0.2 U; however, lower concentrations of collagenase were also used because 0.2 U collagenase produces a signal on the gel that is too strong for evaluation. After electrophoresis, the gel was renatured and then incubated with developing buffer at 37°C for 24 h. After developing, the gel was stained with 0.5% Coomassie Blue R-250 for 30 min and then destained appropriately. Collagenase activity was determined as clear zones or bands by means of densitometry in the FluorChem 5500 Image analyser (Alpha Innotech, San Leandro, CA, USA).

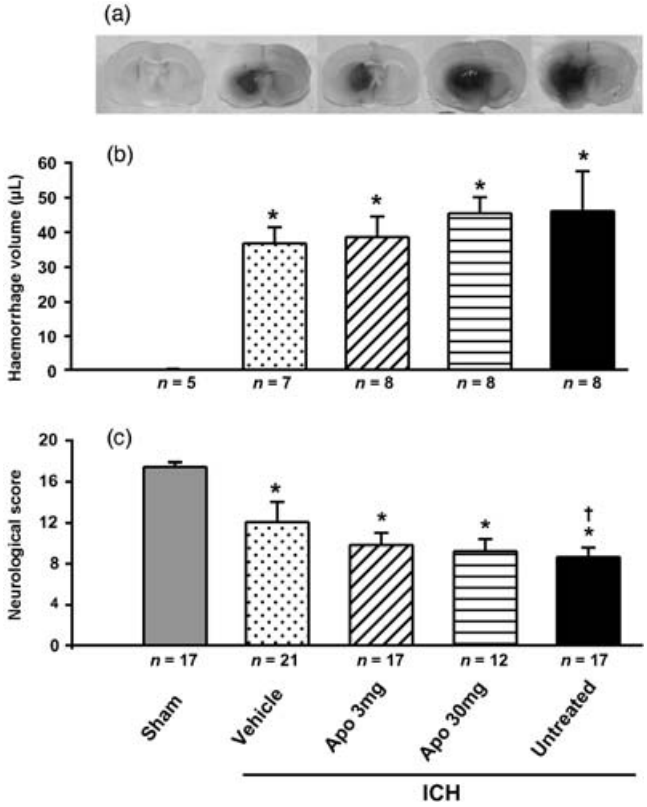

Fig. 1 (a,b) Haemorrhage volume in the ipsilateral hemispheres and (c) neurological scores are shown 24 h after intracerebral haemorrhage (ICH). Collagenase injection induced significant neurological deficit ($P < 0.05$ for ICH *vs* Sham). Apocynin (Apo) treatment did not reduce the haemorrhage volume and did not improve neurological scores. Ethanol significantly reduced neurological deficit in ICH rats ($P < 0.05$ *vs* ICH untreated). $^{*}P < 0.05$ compared with Sham; $^{\dagger}P < 0.05$ compared with ICH + Vehicle.

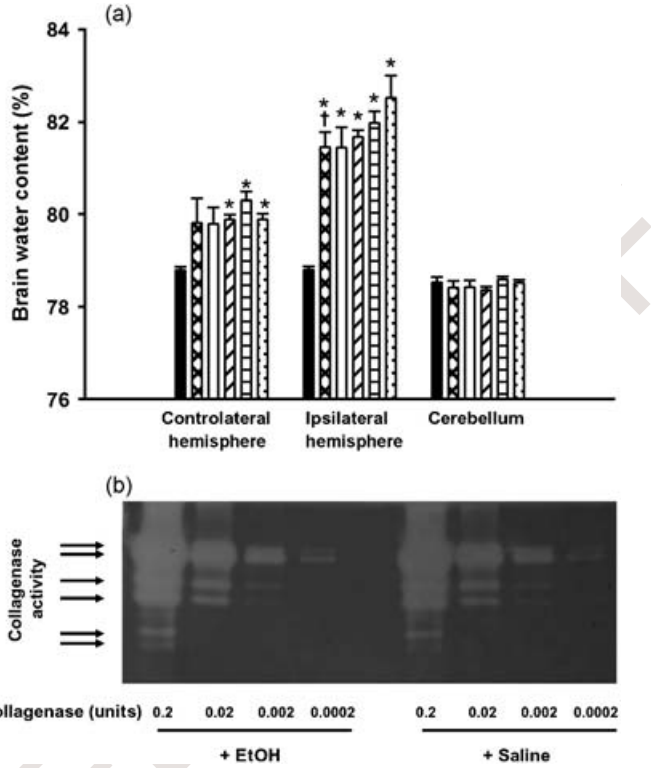

Fig. 2 (a) Brain water content increased markedly after collagenase injection ($^{*}P < 0.05$ intracerebral haemorrhage (ICH) *vs* Sham). Apocynin (Apo) did not reduce brain oedema. Ethanol significantly reduced brain water content in the ipsilateral hemisphere ($^{\dagger}P < 0.05$ *vs* ICH untreated group) and had no effect on the activity of collagenase in the brain of ICH rats (b). (■), Sham ($n = 10$); (▩), ICH + Vehicle ($n = 10$); (□), ICH + 3 mg Apo ($n = 13$); (▨), ICH + 10 mg Apo ($n = 13$); (▤), ICH + 30 mg Apo ($n = 6$); (▦), ICH untreated ($n = 12$).

Statistical analysis

Quantitative data are expressed as the mean±SEM. Statistical significance was verified by ANOVA and Scheffé tests. $P < 0.05$ was considered statistically significant.

RESULTS

Physiological parameters

There were no significant changes in mean arterial pressure, heart rate, blood glucose or blood gas before, during and after ICH induction ($P > 0.05$; data not shown). There was no difference between the effects of 3, 30 and 10 mg/kg (data not shown) apocynin in any of the tests performed.

Haemorrhage volume

Collagenase injection robustly induced ICH in the ipsilateral hemispheres of all ICH rats (Fig. 1a,b; $P < 0.05$ for ICH *vs* Sham, ANOVA). Apocynin did not reduce the haemorrhage volume compared with untreated ICH 24 h after ICH.

Neurological deficits

We observed neurological deficits in all rats with collagenase injection (Fig. 1c; $P < 0.05$, ANOVA). Interestingly, ethanol (0.2 g/kg) significantly reduced neurological deficits in ICH rats compared with the untreated ICH group (12.05 ± 1.99 *vs* 8.65 ± 0.94; $P < 0.05$; Fig. 1c).

Brain water content

The brain water content increased in all rats after collagenase injection ($P < 0.05$ for ICH *vs* Sham; Fig. 2a), especially in the ipsilateral hemisphere ($P < 0.05$ for ipsilateral *vs* contralateral, ANOVA). Apocynin, at 3, 10 and 30 mg/kg, did not reduce brain water content ($P > 0.05$ *vs* ICH untreated, ANOVA). Ethanol, at 0.2 g/kg, significantly reduced brain water content in the ipsilateral hemisphere compared with the ICH untreated group (81.4 ± 0.3 *vs* 82.5 ± 0.4%; $P < 0.05$, ANOVA).

Ethanol on collagenase enzymatic activity

In order to determine whether the reductions in both brain oedema and neurological deficit in ICH rats were due to the inhibition of

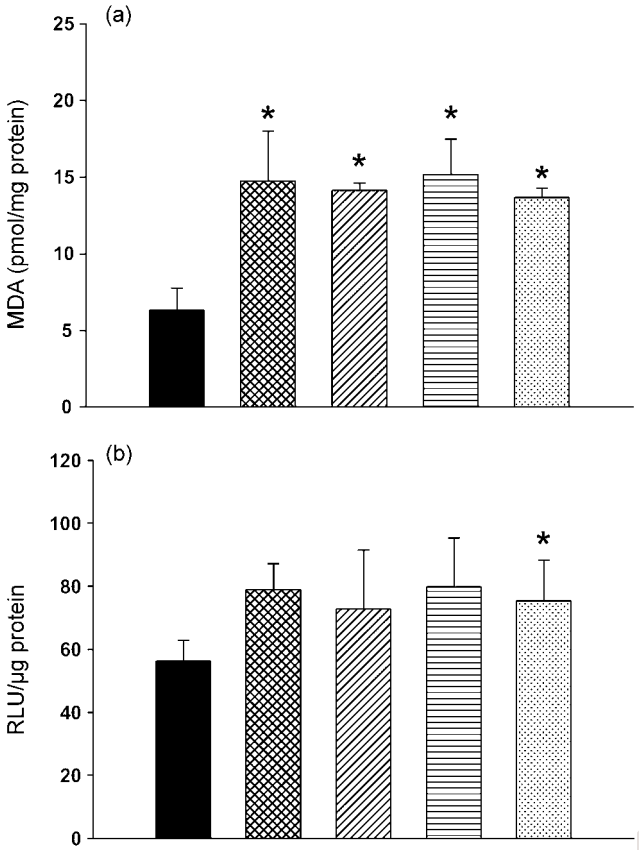

Fig. 3 (a) Collagenase injection increased lipid peroxidation (*$P < 0.05$ *vs* Sham) that was not reduced in the apocynin or vehicle groups. (b) NADPH oxidase activity was significantly increased in untreated intracerebral haemorrhage (ICH) animals; however, neither apocynin nor ethanol reduced NADPH oxidase enzymatic activity. (■), Sham ($n = 4$); (⊠), ICH + Vehicle ($n = 4$); (▨), ICH + 3 mg Apo ($n = 4$); (▤), ICH + 30 mg Apo ($n = 4$); (⬚), ICH untreated ($n = 4$).

collagenase by ethanol, the effect of ethanol on collagenase activity was tested by zymography *in vitro* (Fig. 2b). Figure 2b shows the pattern and intensity of the bands, which indicate no difference in collagenase activity between the ethanol group and the saline group, suggesting that the ethanol in the present study had no effect on the activity of collagenase (0.2 U) in the brain of ICH rats.

Lipid peroxidation

Brain lipid peroxidation was determined by measuring the levels of MDA, lipid peroxidation products, in the ipsilateral hemispheres of Sham and ICH groups (Fig. 3a). Collagenase injection significantly increased brain levels of MDA ($P < 0.05$ *vs* Sham) 24 h after surgery. Neither apocynin nor ethanol reduced lipid peroxidation ($P > 0.05$ *vs* untreated ICH).

NADPH oxidase activity

NADPH oxidase activity in the ipsilateral hemispheres of Sham and ICH groups was measured 24 h after ICH (Fig. 3b). After ICH, NADPH oxidase activity increased significantly ($P < 0.05$ for Sham *vs* ICH-untreated), but no effects of apocynin or ethanol on NADPH oxidase activity were observed.

DISCUSSION

From the present study on ICH, we report an increase in brain NADPH oxidase activity that was accompanied by an increase in lipid peroxidation and brain water content, as well as profound neurological dysfunction. The relationship between the activation of NADPH oxidase and neurological injury has been established previously in a multitude of studies that used either knockout mice or NADPH oxidase inhibitors, including apocynin.[15,21,28,29] In particular, NADPH oxidase seems to play an important role in brain injury induced by ICH because knocking out its *gp91phox* gene produced brain protection in a previous study.[15] Not only was the haematoma volume reduced, but the injury was diminished in the perihematomal tissues. As a result, neurological scores improved.[15] In a recent study, the inhibition of microglial NADPH oxidase by the intrahippocampal injection of diphenylene iodonium reduced thrombin-induced injury in the hippocampus.[30] NADPH oxidase may also be involved in ICH-induced inflammation because microglial NADPH oxidase can amplify pro-inflammatory gene expression *in vivo*.[31]

We had solid expectations of the beneficial effects of pharmacological NADPH inhibition after ICH with apocynin. Apocynin is a cell-permeable intracellular inhibitor of NADPH oxidase that hampers the assembly of its subunits.[16] In a number of *in vitro* experiments, NADPH oxidase inhibitors proved to effectively reduce neuronal death predominantly via the inhibition of apoptosis.[12,28,32] Apocynin reduced cerebral and vascular injury in experimental stroke models at doses similar to those used in the present study.[21,33] However, as opposed to the overwhelmingly positive effects of apocynin treatment for stroke, some other reports pointed out its limited benefits (3 mg/kg, i.p.), such as a short-term protection of selected organs after haemorrhagic shock.[34] In addition, lipopolysaccharide-induced Fe^{2+} toxicity enhancement in cortical neurons was not blocked by an NADPH oxidase inhibitor.[35]

Herein we report that apocynin had no effects on enhanced NADPH oxidase activity, lipid peroxidation and brain water content, as well as the profound neurological dysfunction that occurred after ICH. It should be noted that, in previous studies, the beneficial effects of apocynin were achieved by pretreatment, which has no clinical relevance. Apocynin given 2 h after haemorrhage (as in the present study) could not counter a protein kinase C-mediated, rapid upregulation of enzyme activity.[36] As for the later phase, the presence of hypoxia inducible factor-1 in perihematomal tissues after ICH[37] may suggest the existence of impaired perfusion that makes the brain penetration of apocynin poor. It has been established that perihematomal perfusion is reduced after ICH.[38] Interestingly, the basal NADPH oxidase activity was not reduced by apocynin in the contralateral hemisphere (data not shown) where perfusion was most likely normal. Indeed, partly because of a specific mechanism of apocynin action (i.e. the inhibition of the assembly of NADPH oxidase subunits), apocynin would inhibit the stroke-induced activation of the enzyme rather than the pre-existing activity of enzyme in uninjured tissues. Recently, it has been postulated that, in normal non-phagocytic cells, apocynin induces, rather than suppresses, oxidative stress, although we did not observe such a phenomenon in the present study.[39]

In contrast with the above data, an interesting and promising finding seems to be a slight but significant brain protection conferred upon low-dose ethanol treatment, which reduced oedema in the ipsilateral hemisphere and improved neurological function. Although high doses of ethanol tended to exacerbate brain injury in stroke,[40] at a low dose of 1.2 g/kg, ethanol reduced brain injury that was induced by experimental ischaemia.[41] In addition, a low dose of 0.2 g/kg ethanol was used for the first time against ICH and produced brain protection. In humans, a dose of approximately 0.2 g/kg ethanol can be provided by a standard alcoholic drink (e.g. a can of beer, a glass of wine, one shot of liquor etc.).[42] Low-dose ethanol produces *N*-methyl-D-aspartate (NMDA) receptor antagonism and free radical-scavenging effects.[41,43–46] In the present study, a predominant free radical-scavenging mechanism does not seem likely because no reduction in lipid peroxidation was observed. Therefore, protection against excessive glutamate stimulation seems more likely. It has been suggested that excitotoxicity may have an important role in causing ICH-induced injury[47] and the link between NMDA activation and disruption of the blood–brain barrier is well established.[48,49] Ethanol may directly protect the integrity of endothelial cells that express NMDA receptors.[50]

In conclusion, ICH resulted in the activation of NADPH oxidase and brain injury that cannot be countered by a clinically relevant administration of apocynin. Even though the failure of apocynin to inhibit NADPH oxidase may seem to be a major limitation in the present study, it can also indicate that apocynin should not be considered as a potential therapeutic agent in ICH. Ethanol produced significant brain protection and further studies should be undertaken to optimize the treatment regimen and to elucidate its mechanism.

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