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European Journal of Pharmacology 584 (2008) 100–110
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Using behaviour to predict stroke severity in conscious rats: Post-stroke treatment with 3′, 4′-dihydroxyflavonol improves recovery ☆

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Received 5 June 2007; received in revised form 10 January 2008; accepted 24 January 2008
Available online 12 February 2008

Abstract

Prognostic models are used to predict outcome in stroke patients and to stratify treatment groups in clinical trials. No one has previously attempted to use such models in stroke recovery studies in animals. We have now shown the predictive value of assigning stroke severity ratings, based on behaviours displayed in conscious rats during infusion of endothelin-1 to constrict the middle cerebral artery, on neurological and histological outcomes. The validity of prior stratification of treatment groups according to stroke ratings was tested by assessment of the protective potential of synthetic flavonol, 3′,4′-dihydroxyflavonol (DiOHF). Neurological deficits and performance on the sticky label test were evaluated before and at 24, 48 and 72 h post-stroke. Histopathology was assessed at 72 h. Positive correlations between stroke ratings and neurological deficit scores were found at 24 ($r=0.58$, $P<0.001$), 48 ($r=0.53$, $P<0.001$) and 72 ($r=0.56$, $P<0.001$) h post-stroke, with more severe strokes associated with worse deficit scores. Similar correlations were observed with the sticky label test. Higher stroke ratings also correlated with greater infarct volumes (total infarct volume: $r=0.74$, $P<0.0001$). Treatment with DiOHF (10 mg/kg i.v. given 3, 24 and 48 h post-stroke) significantly reduced infarct volume and restored neurological function in rats with modest stroke ratings ($P<0.01$), but not in rats with high stroke ratings. These results suggest that stroke ratings, based on behavioural assessment as the stroke develops, reliably predict histopathological and functional outcomes and allow stratification of treatment groups. DiOHF given after stroke improves outcomes in moderate strokes, and therefore has cytoprotective potential.

Keywords: Middle cerebral artery; Endothelin-1 model; Prognostic animal model; Flavonols; Cytoprotection

1. Introduction

Despite the continued disappointment from clinical trials of potential neuroprotective drugs in acute stroke there are new clues about how to improve testing of new drugs in pre-clinical animal studies (Feuerstein et al., 2008; Hill, 2007). It is now critical to develop more appropriate animal models of ischemic stroke (Feuerstein et al., 2008). In particular the STAIR guidelines highlight the importance of testing drugs in more than one animal model of stroke prior to clinical trial and to use those that mimic most closely the human condition (STAIR-I, 1999).

Occlusion of the middle cerebral artery mimics closely the cause of the majority of strokes in humans (Mohr et al., 1986), and the rat is the species of choice for it shares a similar cranial circulation to humans (Yamori et al., 1976) and rats have a well documented behavioural profile (Hunter et al., 1998). However recent studies indicate problems associated with the most widely accepted and commonly used model of middle cerebral artery occlusion in rats, that produced by an intraluminal filament. Adverse effects include interruption of blood supply to the

☆ Grant information: this study was supported in part by grants from the National Health and Medical Research Council of Australia (program # 236805) and Neurosciences Victoria. GJD is Principal Research Fellow of NHMRC (# 400303).
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doi:10.1016/j.ejphar.2008.01.046

external carotid artery territory (Dittmar et al., 2003), subsequent hyperthermia (Li et al., 1999), subarachnoid haemorrhage (Schmid-Elsaesser et al., 1998) and damage to the temporalis muscle during surgery resulting in marked weight loss and poorer recovery of motor function (Dittmar et al., 2003). Of particular concern in almost all experimental models is the use of general anaesthesia during induction of stroke, for humans are not usually under anaesthesia when a stroke occurs. Furthermore, barbiturate and inhalational anaesthetics can confound experimental findings due to their own neuroprotective effects (Hagerdal et al., 1978; Bhardwaj et al., 2001), can affect reactive oxygen species (ROS) production (Hagerdal et al., 1978) and cause long-lasting depression of protein synthesis (Fütterer et al., 2004) and ischemic tolerance (Kapinya et al., 2002).

The endothelins are powerful vasoconstrictors that act through 2 receptor subtypes, the endothelin A and endothelin B receptors (Arai et al., 1990). A potent and long lasting constrictor action of endothelin has been described in both animal and human cerebral vasculature, and there is evidence to suggest that it may be involved in the genesis or maintenance of delayed vasospasm following subarachnoid hemorrhage (Zemke et al., 2007), migraine (Tietjen, 2007) and ischemic stroke (Estrada et al., 1994; Patel et al., 1995). Stereotaxic application of endothelin-1 to the middle cerebral artery is now a well established model of focal ischaemic in conscious rats and this model has also been adapted for use in primates (Virley et al., 2004). Although receptors for endothelin are found in non-vascular brain tissue (Nie and Olsson, 1996), endothelin-1 is believed to act at the endothelin A and B receptors on the adventitial surface of cerebral vessels. Thus endothelin-1 injected intraluminally to cerebral arteries induces only minor changes in vessel caliber, unlike abluminal applications (Ogura et al., 1991). Abluminal application of 3 μl of endothelin-1 to the exposed rat middle cerebral artery results in a dose-dependent and reversible vasoconstriction of the artery with up to 80% reduction in cerebral blood flow observed within the first 10 min, similar to that seen with permanent occlusion of the proximal middle cerebral artery — a standard model of focal ischaemia in rats (Macrae et al., 1993; Sharkey et al., 1993; Sharkey and Butcher, 1995). The endothelin model is the only model in which focal cerebral ischemia ('stroke') is induced in the absence of anaesthesia, and in which spontaneous reperfusion occurs (Sharkey et al., 1993). However a disadvantage of this model is that it is not practical to measure cerebral blood flow in conscious rats to determine the duration of ischemia (Bogaert et al., 2000), and thus it has been difficult to determine the initial severity of stroke in this model.

Prognostic clinical approaches such as the use of the Scandinavian Stroke Scale, allow prediction of functional outcome and survival in stroke patients in order to support clinical management and to correctly stratify treatment groups in clinical trials (Counsell et al., 2002). The use of a similar approach in experimental animals has not previously been attempted. By administering endothelin-1 to constrict the middle cerebral artery it is possible to observe behavioural responses during the induction of stroke because the rats are conscious. We and others have observed behavioural responses including circling in the direction contralateral to the occluded artery, and clenching and dragging of the contralateral forepaw (Sharkey et al., 1993, Callaway et al., 1999; Bogaert et al., 2000). Based on these responses we have assigned a rating scale of stroke severity. The present study was undertaken to determine the predictive value of behavioural observations and ratings made during induction of stroke using the endothelin-1 model in conscious rats, on both histological and behavioural outcomes.

We have chosen to study flavonols as potential cytoprotective agents. Flavonols are clinically well tolerated, highly lipid soluble antioxidants that easily permeate biological membranes and scavenge intracellular, as well as extracellular reactive oxygen species (Pietta, 2000). We have recently shown that 3′, 4′-dihydroxyflavonol (DiOHF), given before reperfusion of the ischemic myocardium in sheep, greatly reduces infarct size, myocardial injury and helps restore coronary blood flow (Wang et al., 2004). In addition, DiOHF enhances nitric oxide bioavailability and improves vascular function after ischemia and reperfusion injury in rat hindquarters (Chan et al., 2003). We therefore set out to assess the protective potential of DiOHF in stroke using behavioral responses to stratify rats into groups based on stroke severity (as discussed above), prior to assessment of recovery of neurological functions and morphologically mapped brain damage.

2. Materials and methods

2.1. Surgical preparation

All experiments were performed in accordance with the guidelines of the National Health & Medical Research Council of Australia Code of Practice for the Care and Use of Animals for Experimental Purposes in Australia, which complies with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the United States National Institutes of Health. Male Hooded Wistar rats (280–340 g, $n=69$) were anesthetised with pentobarbital sodium in a volume of 0.6 ml (60 mg/kg i.p.). A 23-gauge stainless steel guide cannula was stereotaxically implanted into the piriform cortex 2 mm dorsal to the right middle cerebral artery (0.2 mm anterior, −5.2 mm lateral and −5.9 mm ventral) according to the method of Sharkey and colleagues (1993) and described previously (Callaway et al., 1999). For rats receiving acute drug treatment, an intravenous (i.v.) catheter was inserted into the jugular vein. Rats were housed individually on a 12 hour day/night cycle at a temperature of 18–22 °C and allowed to recover for 5 days before induction of stroke.

2.2. Stroke induction

A total of 64 rats were used for stroke induction. Constriction of the right middle cerebral artery was induced in conscious rats by perivascular administration of endothelin-1 (American Peptide Company, Inc. CA, USA) (60ρmol in 3 μl of saline over 10 min) (Callaway et al., 1999). Rats were placed in a clear Plexiglass box for observation during endothelin-1 injection. During stroke induction we observed counter-clockwise circling, clenching and dragging of the contralateral forepaw, validating the correct placement of the cannula. Stroke severity was scored 1 to 5 based on these responses, 5 being the most severe (Table 1). Behavioural changes occurred

Table 1
Changes in behaviour upon ET-1 injection in the conscious rat indicates stroke intensity that can be rated 1-5

Time	Observed behaviour	Stroke rating
0–2 min	Systematic grooming, teeth chattering	1
	Tongue poking, licking bedding	
	Raised contralateral forepaw	
	Raised and clenched contralateral forepaw	
2–4 min	Bitting cage and bedding	2
	Head turned to contralateral direction	
	Head bobbing in contralateral direction	
	Spasmodic contralateral turns (not continuous)	
5–10 min	Continuous contralateral turning	3
	Chin rubbing	
10–30 min	Continuous ipsilateral circling	4
	Ipsilateral forepaw clenched	
	Chin rubbing	
	Forepaw shuffling/digging whilst turning	
>30 min	Still circling after 60 minutes	5
	Loss of balance on rearing/walking	
	Loss of righting reflex	

within 2 to 10 min of the commencement of the endothelin-1 infusion and were observed and rated over 60 min. After this time rat behaviour returned to normal, although some rats continued to circle for up to 2 h following endothelin-1 infusion in those ranked as having a severe stroke rating of 5. Rats that did not display any behavioural change over 60 min were deemed not to have suffered a stroke and were excluded from the study. Control rats ($n=5$) underwent cannula implantation and saline infusion instead of endothelin-1 to demonstrate that saline injection itself does not induce vasoconstriction and cerebral infarction, as previously described (Callaway et al., 1999, 2000). Rectal temperatures were taken with a thermistor probe, prior to stroke and at 30- or 60-min intervals for 3–5 h after stroke.

2.3. Drug treatment

In the drug treatment study, rats were assigned a stroke severity score during stroke induction and divided into DiOHF or vehicle treatment groups. In each treatment group stroke ratings were equally matched in order to ensure that stroke severity was evenly represented across treatments. DiOHF (10 mg/kg, dissolved in 20% DMSO, 40% polyethyleneglycol and 40% sterile water) ($n=13$) or vehicle ($n=11$) was given intravenously 3 h post-stroke onset and then again at 24 and 48 h after stroke (total of 3×10 mg/kg). The dose of DiOHF was chosen based on preliminary investigations where 3 mg/kg showed some reduction in infarct size and 10 mg/kg produced considerable reduction (unpublished data).

2.4. Assessment of functional outcome

All behavioural tests were conducted prior to any procedures (pre-surgery, day 1), immediately prior to endothlin-1-induced middle cerebral artery constriction (pre-ischemia, day 6) and 24, 48 and 72 h after endothlin-1-induced middle cerebral artery. The behaviour of each rat was compared to pre-stroke, thus each rat acted as its own control. All rats were coded so that the investigator was blinded to treatment condition. Neurological abnormalities were evaluated with the use of a neurological deficit score based on detection of abnormal posture and hemiplegia, and asymmetry was evaluated using the sticky label test as previously described (Callaway et al., 1999).

2.5. Quantification of ischemic damage

Rats were decapitated 72 h after ischemia and their brains were removed and frozen in liquid nitrogen and stored at −80 °C. Coronal cryostat sections (16 μm) were cut at eight predetermined coronal planes throughout the brain from −3.2 to 6.8 mm relative to Bregma. Infarct was measured in triplicate unstained sections using our previously reported novel method (Callaway et al., 2000). Total infarct volume was calculated by integrating the cross-sectional area of damage at each stereotaxic level with the distances between levels. The influence of oedema on the infarct area was corrected by applying the following formula: (area of normal hemisphere/area of infarcted hemisphere)×area of infarct. Slides were also coded so that the investigator was blinded to treatment condition.

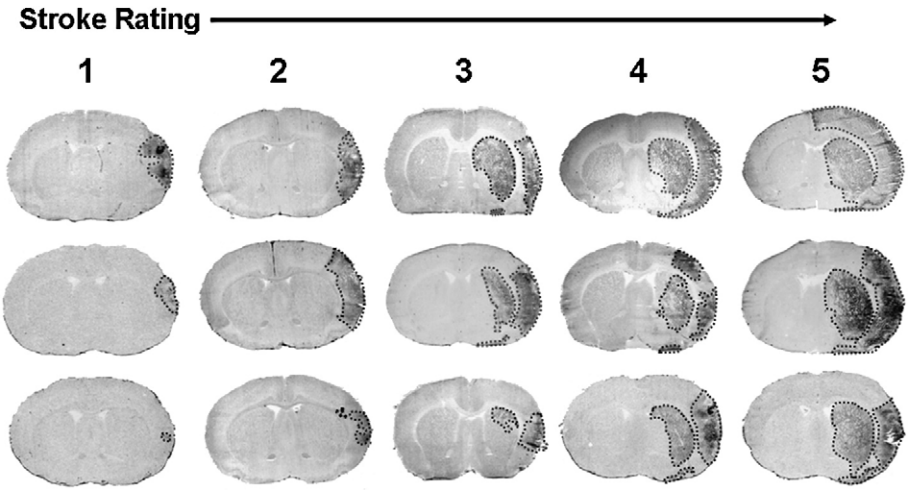

Fig. 1. Stroke infarct images from several rats at each stroke rating 1–5.

Table 2
Neurological deficit score at 0, 24, 48 and 72 h after ischemia for all stroke ratings and following treatment with DiOHF

Stroke rating	N	Hours after Ischemia			
		Pre-stroke	24	48	72
Sham	5	0.0±0.0	0.6±0.6	0.0±0.0	0.0±0.0
ET-1, all stroke ratings	40	0.4±0.1	3.9±0.5[a]	4.6±0.5[a]	5.1±0.5[a]
1	4	0.3±0.3	1.3±0.3	2.0±0.7	1.5±0.6
2	7	1.0±0.6	1.7±0.8	2.9±0.7	3.7±0.8
3	9	0.2±0.1	3.7±1.1[a, b]	4.6±0.6[a, b]	5.1±0.6[a, b]
4	15	0.3±0.2	4.3±0.7[a, b, c]	4.5±0.7[a, b, c]	5.2±0.8[a, b]
5	5	0.2±0.2	8.4±1.6[a, b, c, d, e]	9.6±2.3[a, b, c, d, e]	9.7±1.9[a, b, c, d, e]
Vehicle (all ratings)	11	0.2±0.1	4.5±0.9[a]	5.9±0.9[a]	6.5±0.9[a]
DiOHF (all ratings)	13	0.3±0.1	4.5±0.8[a]	5.3±0.9[a]	6.1±0.9[a]
Vehicle (ratings 2–3)	6	0.2±0.2	3.1±1.1[a]	5.3±0.8[a]	6.3±0.5[a]
DiOHF (ratings 2–3)	6	0.2±0.2	3.0±0.8[a]	2.8±0.6[a, f]	4.1±0.9[a, f]

Values are mean±S.E.M. for ET-1 infused rats and sham rats. [a]$P<0.05$ compared with sham rats and pre-stroke scores (ANOVA). [b]$P<0.05$ compared with stroke rating 1, [c]$P<0.05$ compared with stroke rating 2, [d]$P<0.05$ compared with stroke rating 3, [e]$P<0.05$ compared with stroke rating 4 at the same time interval following ET-1 injection, [f]$P<0.05$ compared with vehicle treated rats (ANOVA).

2.6. Statistical analyses

All statistical analysis was conducted in consultation with Prof John Ludbrook Director Biomedical Statistical Consulting Service Pty. Ltd., Carlton North, Victoria, Australia. Physiological and neurological outcome data were analysed by two-way ANOVA for comparison between sham-operated and endothelin-1-injected stroke groups and between DiOHF and vehicle-treated rats. For analysis of physiological data or neurological outcome following endothelin-1 injection a one-way RM-ANOVA (treatment x h after stroke) was performed. The sticky label test data was analysed by two-way RM ANOVA with 2-factor repetition (side x h after stroke) to compare latencies in the ipsilateral and contralateral forepaws over time. One sample *t*-tests were used to determine significance of asymmetry from chance or 0.05. For comparison between stroke rating and infarct volume, or stroke rating and neurological outcome at each time interval following stroke, a one-way ANOVA was performed. Individual comparisons were made using Tukey's test for all analyses where ANOVA yielded a significant result. Values are presented as mean±S.E.M. A two-sided value of $P \leq 0.05$ was considered statistically significant. Data were analysed using SigmaStat 2.03 (SPSS Inc. Chicago, IL). Finally, to test for correlation between stroke rating and infarct volume, or stroke rating and neurological outcome, the Pearson product-moment coefficient, *r*, for ordinal values was determined using GraphPad Prism, version 4 (GraphPad Software Inc., San Diego CA).

3. Results

3.1. Stroke rating

Rats showed neurological behavioural deficits indicative of stroke within 2–10 min of endothelin-1 injection, but not after injection of saline in the sham group. These deficits included specific behavioural responses such as clenching and failure to extend the contralateral forepaw, and circling in the direction contralateral to the occlusion. Behavioural responses observed at the time of stroke could be rated based on their degree and severity (Table 1, Fig. 1). Grooming behaviour preceded circling and was observed in almost all rats. Grooming occurred in a stereotypical manner with facial grooming being followed by full body grooming in one continuous movement. Other behaviours such as teeth chattering, biting of the cage and bedding or tongue poking, were observed less frequently. In the drug treatment study, rats had stroke severities ranging from 2 through 5 as a stroke rating of 1 was never observed in this group of rats.

3.2. Physiological variables

All rats, including sham-injected rats, lost weight (~10% of starting weight) after surgery. Weight loss in the sham-group did not differ from rats in the endothelin-1 group following surgery and sham rats gained weight over time. Rats receiving endothelin-1 injection had significant weight reduction 24, 48 and 72 h post-stroke ($P<0.01$, ANOVA with repeated measures for hrs after stroke) similar to that previously reported (Callaway et al., 1999). Rectal temperature prior to surgery in both groups was within normal physiological limits. Core temperature was not significantly altered after intravenous treatment with DiOHF or vehicle at 3 h after the onset of stroke, indicating that any effects seen with DiOHF on infarct size and functional outcome could not be attributed to alterations in thermoregulation (data not shown).

3.3. Functional assessment including DiOHF treatment

Endothelin-1-injected rats exhibited significantly higher neurological deficit scores 24, 48 and 72 h post-stroke when compared to sham injected controls and pre-stroke scores ($n=40$) ($P<0.001$, ANOVA, Table 2). When analysed for individual stroke rating, rats with higher stroke ratings exhibited significantly higher neurological deficit scores when compared with lower stroke rating groups at 24, 48 and 72 h post-stroke ($P<0.001$, ANOVA) (Table 2). This was confirmed by a positive correlation between stroke rating and neurological deficit score 24 ($r=0.58$, $P<0.001$), 48 ($r=0.53$, $P<0.001$) and

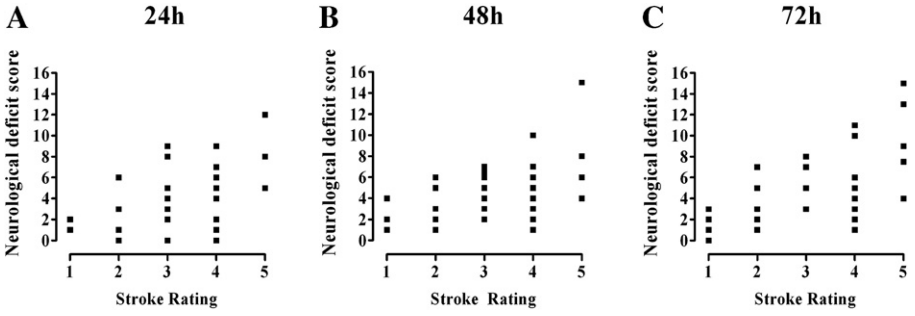

Fig. 2. Scatter plots depicting correlation between stroke rating and neurological deficit score 24 (A), 48 (B) and 72 h (C) after ischemia. A significant correlation was found between stroke rating and neurological deficit score at 24 ($r=0.58$, $P<0.001$), 48 ($r=0.53$, $P<0.001$) and 72 ($r=0.56$, $P<0.001$) h post-stroke (Pearson product moment correlation coefficients).

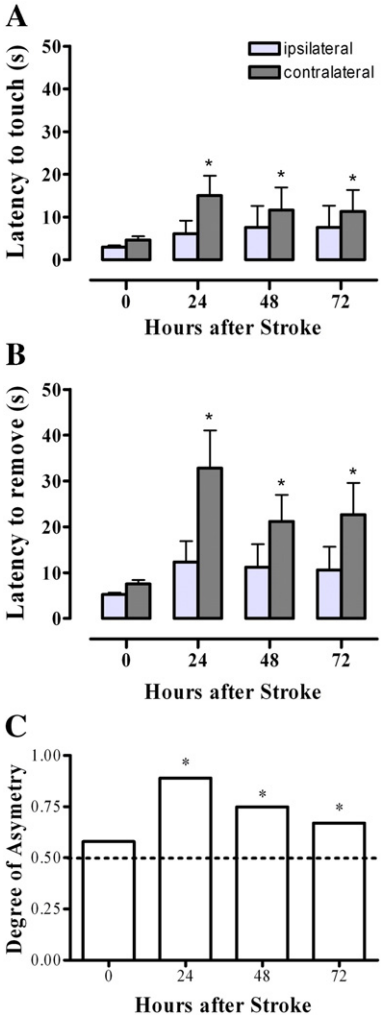

Fig. 3. Latency to touch and remove a stimulus (sticky label) on the contralateral (stroke affected) forelimb compared with the ipsilateral forelimb when assessed 24, 48 and 72 h after stroke (A and B). Data presented as mean±S.E.M. of time taken to touch each stimulus and then remove the stimulus for $n=40$ rats (A and B). *$P<0.05$ compared with 0 h score in the ipsilateral forelimb at the same time measurement (RM ANOVA). The degree of asymmetry was determined by scoring which limb, impaired (contralateral, score=1) or unimpaired (ipsilateral, score=0), was touched first (C). The dotted line represents the chance level of 0.5. Data was analysed for significant difference from chance by one sample t-test. *$P<0.05$ compared with chance.

72 ($r=0.56$, $P<0.001$) h post-stroke, with more severe strokes correlating with worse deficit scores (Fig. 2). Treatment with DiOHF had no overall effect on neurological deficit score following stroke when all stroke ratings were combined and compared to vehicle control ($n=13$) (Table 2). However, when analysed based on predicted outcomes, DiOHF treatment significantly reduced neurological deficits in animals assigned stroke ratings of 2 and 3 when matched to vehicle-treated rats with the same stroke scores ($n=6$) ($P<0.05$, ANOVA) (Table 2).

Following stroke in all rating groups, latency to touch and remove sticky labels from the stroke affected contralateral forepaw was significantly increased when compared with the ipsilateral side ($P<0.001$, two-way RM-ANOVA with 2-factor repetition, h after stroke and side, followed by Tukey's test) (Fig. 3A and B). A significant degree of asymmetry was found between the ipsilateral and contralateral side at all times after stroke, with rats showing preference to touch the unimpaired side, prior to turning attention to the impaired forelimb (Fig. 3C). Positive correlations between stroke rating and time to touch and remove sticky labels on the impaired contralateral side were found at all times after stroke (Table 3).

When compared to vehicle-treated rats, treatment with DiOHF (3×10 mg/kg) was not effective at reducing latencies to touch and remove sticky labels when rats from all stroke ratings were combined ($n=13$) (Fig. 4A–D). In contrast, DiOHF completely abolished the increase in latency to touch and remove sticky labels on the contralateral side in rats with stroke rating scores of 2 and 3 as compared to vehicle-treated rats with the same stroke scores ($n=6$) (Fig. 4E-H).

Table 3
Correlations between stroke rating and time taken to touch and remove sticky labels post-stroke

Time after stroke	Touch		Remove	
	Contralateral	Ipsilateral	Contralateral	Ipsilateral
24 h	0.44[b]	0.21	0.46[b]	0.33
48 h	0.44[b]	0.24	0.50[b]	0.31
72 h	0.40[a]	0.28	0.53[b]	0.32

Data shown are Pearson product moment correlation coefficients determined for comparisons of latency to touch or remove sticky tapes on the contralateral or ipsilateral forepaws and the ratings given to rats during stroke. [a]$P<0.05$, [b]$P<0.01$.

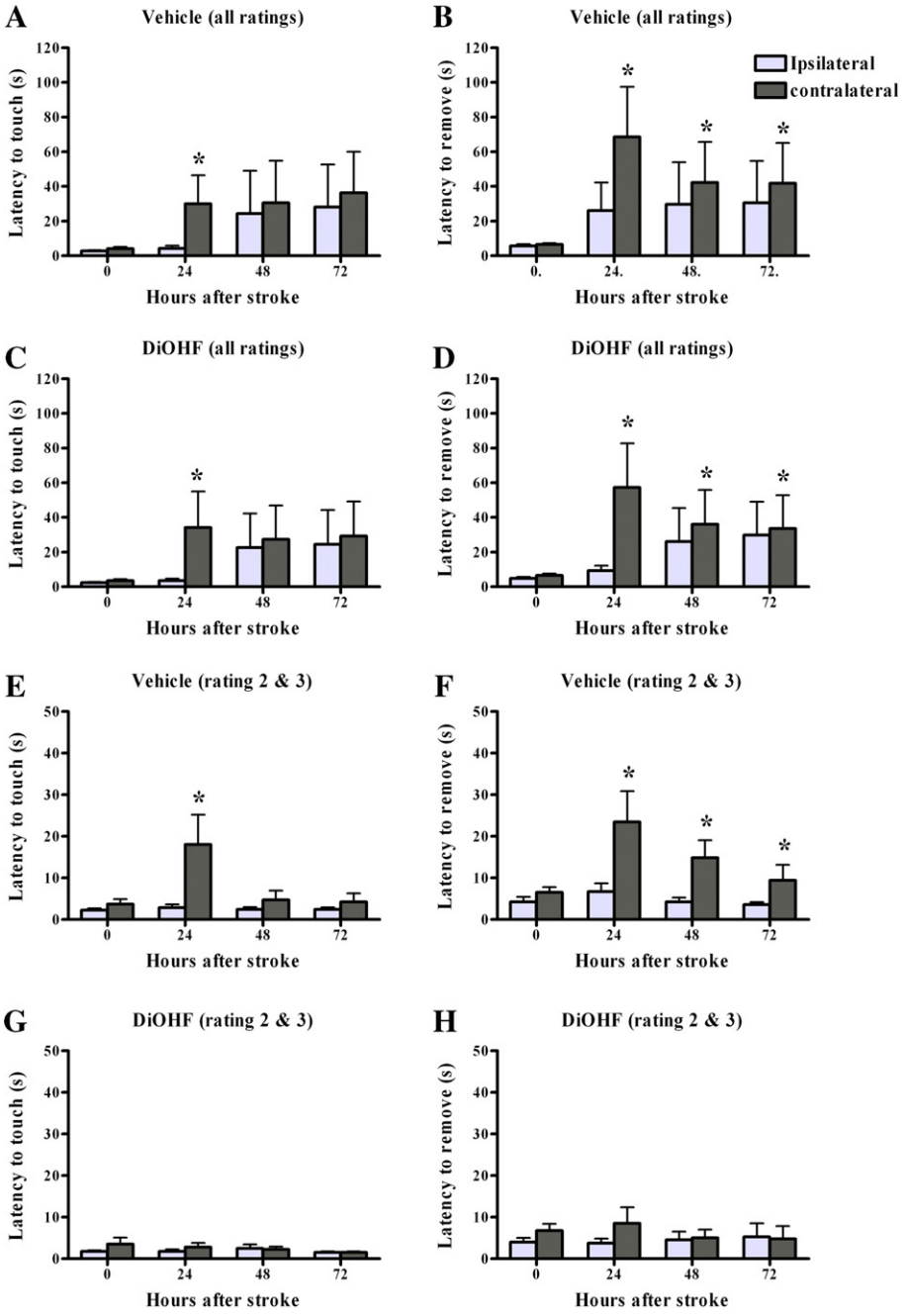

Fig. 4. Latency to touch and remove a stimulus (sticky label) on the contralateral (stroke affected) forelimb compared with the ipsilateral forelimb when assessed 24, 48 and 72 h after stroke. Data presented as mean ± S.E.M. of time taken to touch (A,C,E,G) and remove (B,D,F,H) each stimulus for vehicle treated rats ($n=11$) (A, B); DiOHF treated rats ($n=13$) (C, D); vehicle treatment in rats with stroke ratings 2 and 3 ($n=6$) (E, F); DiOHF treatment in rats with stroke ratings 2 and 3 ($n=6$) (G, H). *$P<0.05$ compared with 0 h score in the ipsilateral forelimb at the same time measurement (RM ANOVA).

3.4. Histopathological analyses

Histopathology 72 h after stroke revealed varying degrees of damage in the parietal, insular, and frontal cortex, as well as in the dorsolateral striatum, which sometimes extended throughout the striatum (Table 1). Sham injected rats showed only localized damage associated with the tract of the guide cannula. When analysed for each individual stroke rating, significant differences in infarct volumes were found for each of the stroke rating groups for total infarct volume, volume of damage through cortex and volume of damage through striatum ($P<0.001$, ANOVA, stroke rating × infarct volume, Fig. 5A–C). This was confirmed by significant correlations between infarct volume and stroke rating for all regions (total: $r=0.74$, $P<0.0001$; cortex: $r=0.66$, $P<0.0001$; and striatum: $r=0.79$, $P<0.0001$), with higher stroke ratings correlating to greater infarct volumes (Fig. 5D–F).

Treatment with DiOHF significantly reduced infarct area through the cortex, but not striatum, at several stereotaxic levels when data from rats of all stroke ratings were pooled and

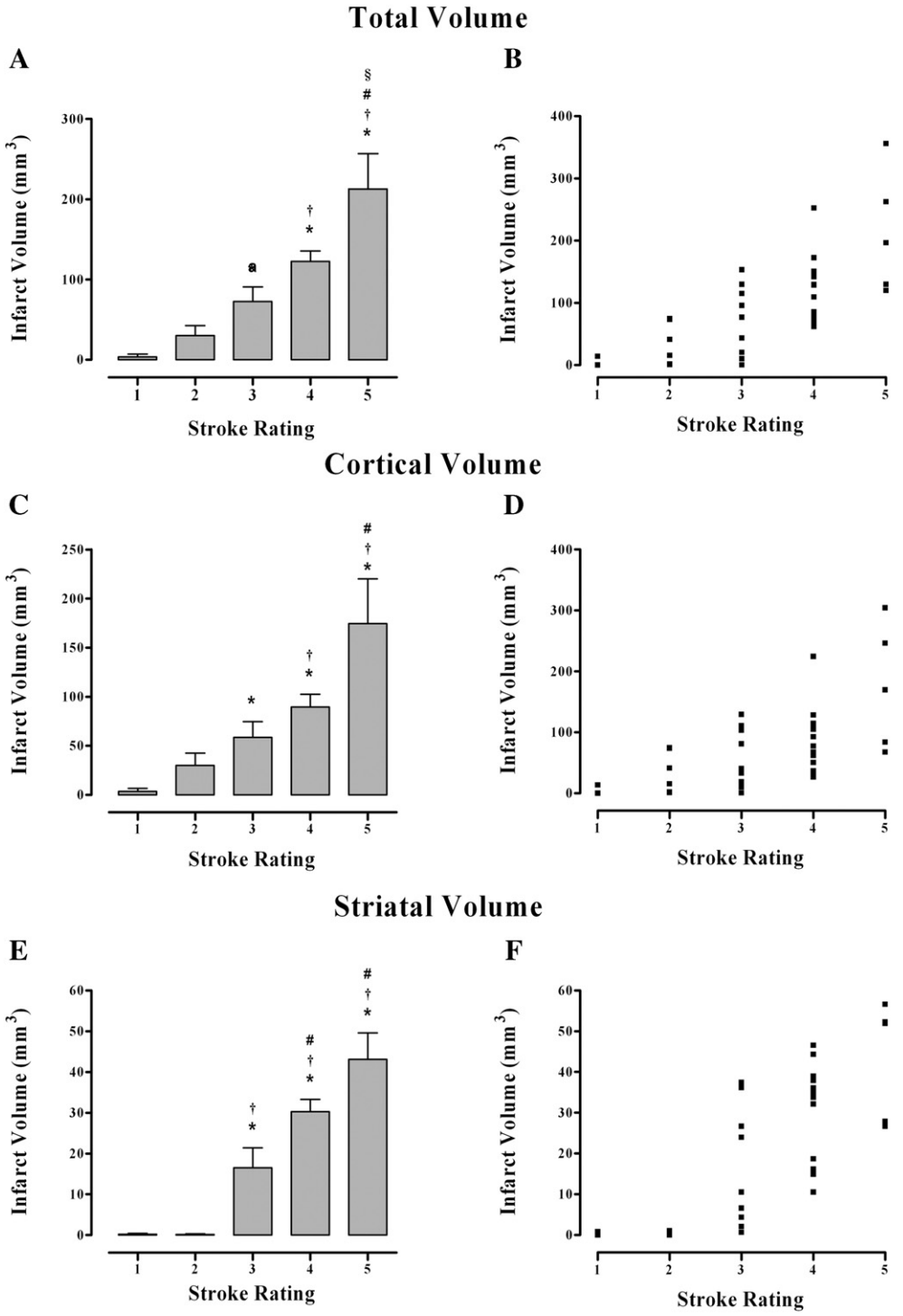

Fig. 5. Infarct volume for each stroke rating group: total volume of damage (A), volume of damage in cortex (C) and volume of damage in striatum (E). Data are presented as mean±S.E.M. for $n=4$ stroke rating 1; $n=7$ stroke rating 2; $n=9$ stroke rating 3; $n=15$ stroke rating 4; $n=5$ stroke rating 5. *$P<0.05$ compared with stroke rating 1, †$P<0.05$ compared with stroke rating 2, #$P<0.05$ compared with stroke rating 3, §$P<0.05$ compared with stroke rating 4 (ANOVA). Scatter plots depicting correlation between stroke rating and total volume of damage (B), volume of damage in cortex (D), and volume of damage in striatum (F). A significant correlation was found between stroke rating and total volume of damage ($r=0.74$, $P<0.0001$), volume of damage in cortex ($r=0.66$, $P<0.0001$) and volume of damage in striatum ($r=0.79$, $P<0.0001$) (Pearson product moment correlation coefficient).

compared to vehicle treatment ($n=13$) ($P<0.01$, ANOVA) (Fig. 6A and B). However, in rats with modest stroke ratings of 2 and 3, DiOHF treatment significantly reduced damage through the cortex, and virtually eliminated striatal damage, as compared to vehicle-treated rats with the same stroke ratings ($n=6$) (Fig. 6C and D). Infarct volume in the lower stroke rating groups was reduced by 44% in the cortex and 92% in the striatum following treatment with DiOHF, although this did not

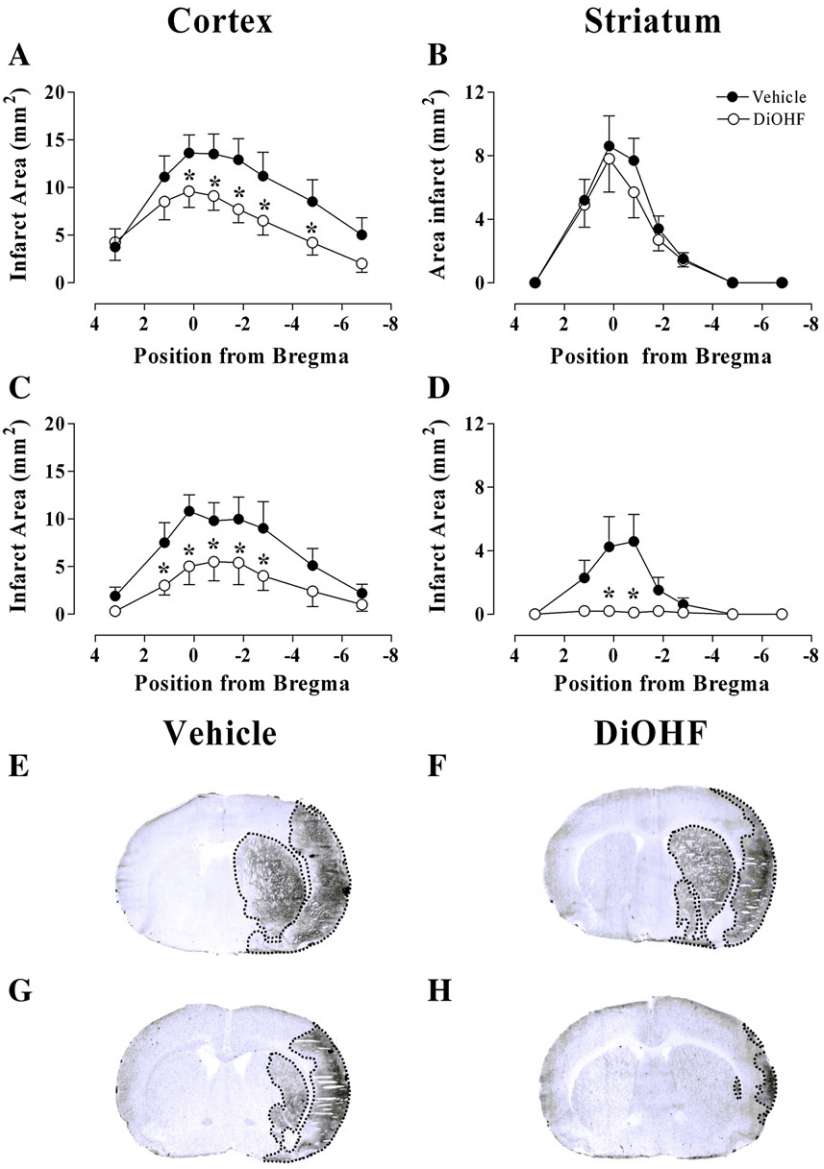

Fig. 6. Effect of delayed administration of DiOHF ($n=13$) or vehicle ($n=11$) on infarct area in cortex and striatum in all stroke rats (A and B) and in rats with moderate stroke ratings (scores 2 and 3) ($n=6$) (C and D). Data are presented as±S.E.M. of infarct area measured at 8 predetermined coronal planes through the forebrain. *$P<0.05$ compared with vehicle-treated control rats (ANOVA). Images generated from unstained forebrain sections depicting the effect of delayed administration of vehicle (E and G) or DiOHF (F and H) on infarct area in cortex and striatum in all stroke rats (E and F) and in rats with moderate stroke ratings (scores 2 and 3) (G and H). The dotted line highlights infarct area.

quite attain statistical significance ($P=0.06$ and 0.09 respectively, ANOVA).

4. Discussion

Here we demonstrate for the first time that in the conscious rat it is possible to predict accurately both histological and functional outcomes based on behavioural changes observed during infusion of endothelin-1 to constrict the middle cerebral artery. In addition, we have demonstrated the usefulness of stratifying rats into treatment groups using stroke ratings based on initial behaviours, to test the outcomes of synthetic flavonol treatment across all stroke outcomes. Using this prognostic model we demonstrate that stratification of DiOHF treatment revealed variable degrees of histological and functional protection across different stroke severities. Prognostic models are used in human patients upon clinical presentation to predict outcome and to assist in assignment and management of treatment (Counsell et al., 2002), but such stratification has not previously been attempted in animal studies. This is an important factor to be considered when determining the clinical potential of any cytoprotective agent.

It has been suggested that a possible reason for the failure to translate positive results in animal studies to clinical trials may be due to assignment of inappropriate patients to the trails, based on factors such as stroke severity, stroke type, location of lesion and whether the affected tissue is responsive to the trial drug (Fisher, 2003). Indeed the extent of the benefit of

therapeutic agents in human strokes appears to be dependent on the type and severity of the stroke (Murin et al., 2001; STAIR-I, 1999). Similar problems are likely to occur in pre-clinical studies where variability does occur in the severity of the strokes between animals, but have not been adequately accounted for in the data. It is clear that an unequal number of rats with mild strokes in a treatment group compared to a vehicle control group could wrongly lead to a positive outcome for a protective drug treatment. Such problems in the past may have contributed to inappropriate positive findings for neuroprotective agents in animal studies. Making assessment of the stroke rating and hence predicted infarct volume before assigning treatment groups enables the experimenter to assign rats from each rating equally to treatment and control groups leading to a more balanced experimental outcome. The present stroke rating scale could potentially be applied to the commonly used intra-luminal filament model and to the embolic stroke model if isofluorane anaesthesia were interrupted within minutes after stroke induction.

In the present study we were able to characterise differential effects of the synthetic flavonol DiOHF on histological and functional outcome in rats with differing stroke severity. Although delayed treatment with DiOHF reduced damage in the cortex across all stroke severities, this was not the case in the striatum and there was no significant effect on neurological outcomes. However, in a sub-group of rats with moderate stroke severity, DiOHF markedly reduced damage to the striatum, as well as cortex, and importantly this effect translated to improvement in neurological function. A reliable rating scale, such as that reported here, allows stratification of groups into mild, moderate and severe stroke, and hence animals can be assigned to treatment groups based on stroke severity prior to assessment of neurological function and histological outcome. Such pre-treatment assignment enables potential neuroprotective agents to be reliably tested across all stroke severities, at the same time enabling further subgroup assessment. Currently, the endothelin-1 model of stroke is the only experimental model in which stroke is induced without anaesthesia, allowing stroke severities to be graded based on changes in behaviour at the time of stroke in a similar manner to that which occurs in stroke patients.

As in humans, the severity of stroke can have a marked impact on the efficacy of treatment (The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group, 1995; Fagan et al., 2003). In the present study, rats with higher stroke ratings had infarcts involving most of the affected hemisphere, therefore limiting the amount of the brain that might be salvaged with any agent. Under such circumstances the ischemic penumbra may be greatly reduced, thus limiting the amount of salvageable tissue where ischemic damage can be reversed prior to cell death. Indeed, treatment with DiOHF in the present study was only marginally effective in rats with severe stroke ratings. It is now possible to identify the ischemic penumbra by MRI in human patients using mis-match between perfusion-weighted imaging abnormality and a diffusion-weighted imaging lesion (Butcher et al., 2005). Therefore, assessment of salvageable tissue in individual stroke patients should be considered prior to assigning treatment in clinical trials, which may result in better patient outcomes.

It is also important to assess long-term neurological deficits following stroke in animals (Dittmar et al., 2003; STAIR-I, 1999) and it remains to be seen whether the present rating scale can predict outcome beyond 3 days. Stroke rating correlated well with infarct volumes, neurological deficit scores and with asymmetry in the sticky label test up to 72 h after stroke. Behavioural and histological endpoints have often been dissociated in animal models of stroke (Windle and Corbett, 2005) probably because of difficulties in relating such diffuse damage as occurs in ischemic stroke to a single behavioural measure. Unilateral lesions to areas of the brain controlling sensorimotor functions, especially those involving the forelimb regions, result in a bias towards removing sticky labels from the unimpaired limb (Schallert and Woodlee, 2005). This finding was confirmed in the present study and indeed there were strong correlations between stroke rating and latencies to touch and remove sticky labels from the impaired limb. Some discrepancies in latencies recorded between non-treated animals ($n=40$) and vehicle treated animals ($n=11$) are difficult to explain highlighting the need to use more than one behavioural measure to determine effects on outcome (STAIR-I, 1999). The present data indicate that performance on simple neurological deficit assessment tests can also be predicted accurately by stroke ratings assigned during stroke induction, and that both correlate strongly with the histological outcome.

An ideal therapeutic agent for stroke should offer significant cytoprotection with a wide therapeutic time window, and in recent studies we and others have found that delayed treatments (up to 5 h) with radical-scavenging compounds are protective in experimental animal models of ischemic stroke (Callaway et al., 1999; Marshall et al., 2003). Polyphenolic flavonols are an important group of antioxidants ubiquitous in fruits, vegetables and herbs, and recent studies have shown neuroprotective effects of naturally occurring flavonoids following stroke (Dajas et al., 2003; Gupta et al., 2003). Phenolic compounds have significant antiinflammatory effects (Jiang and Dusting, 2003; see review Jiang et al., 2008) and flavonols have been reported to cause vasorelaxation (Chan et al., 2000), scavenge superoxide (Chan et al., 2003; Wang et al., 2004) and peroxyl radicals (Dugas et al., 2000), as well as inhibit a variety of enzymes responsible for superoxide generation (Pietta, 2000; Jiang et al., 2008). The synthetic flavonol, DiOHF, has 3-hydroxyl groups in the heterocyclic ring and a catechol group in ring B, which favours anti-oxidant activity (Crozier et al., 2000). Furthermore, DiOHF is a significantly more potent vasorelaxant than many naturally occurring flavonols such as quercetin and chrysin (Woodman and Chan, 2004). Given that oxygen radical levels are dramatically increased following reperfusion after stroke (Murin et al., 2001) and that early restoration of blood flow improves outcome in stroke patients (The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group, 1995), it is possible that DiOHF exerts its protective effects through antioxidant and vasodilator activities that limit injury associated with vascular reperfusion. Indeed, DiOHF is strongly cytoprotective in other models of ischemia and reperfusion

injury, which appears to result from attenuating superoxide levels and improving blood flow to the ischemic region (Chan et al., 2003; Wang et al., 2004). Although the precise mechanism through which DiOHF positively influences infarct size and neurological function in ischemic stroke remains to be determined, DiOHF could potentially target numerous anti-ischemic events such as improving cerebral blood flow post-ischemia as well as preventing oxidative stress, especially that derived from NADPH oxidase (Jiang et al., 2008), and could be used in combination with existing stroke therapies.

In conclusion, our findings suggest that rating behaviours observed during stroke induction by endothelin-1 infusion in conscious rats reliably predicts total, cortical and striatal infarct volume as well as neurological deficits up to 72 h following stroke. Furthermore, we have demonstrated the importance of stratifying animals, based on stroke rating, by demonstrating differential effects of DiOHF on mild to moderate strokes compared with severe strokes. This study highlights the importance of testing new protective agents against all stroke severity in pre-clinical studies in order to determine potential clinical value. Furthermore, the present study confirms the cytoprotective potential of synthetic flavonoids in the development of drugs that multitarget anti-ischemic events.

Acknowledgments

This study was supported in part by grants from the National Health and Medical Research Council of Australia (program # 236805) and Neurosciences Victoria. GJD is Principal Research Fellow of NHMRC (# 400303). We thank Prof John Ludbrook MD DSc ChM BMedSc FRCS FRACS AStat, Director Biomedical Statistical Consulting Service PTY LTD, Carlton North, Victoria, Australia for advice on statistical analyses.

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