The Effect of Nicardipine on Neuronal Function Following Ischemia

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SUMMARY In cerebral ischemia, it has been proposed that calcium influx into neurons results in irreversible cellular injury during reperfusion. We administered nicardipine, a dihydropyridine calcium entry blocker, by continuous subcutaneous infusion to twenty five rats beginning before (PR) or following (PO) ischemia, and compared somatosensory evoked potentials (SEPs) to twenty eight ischemic control animals. Comparable ischemic cellular changes were seen in the hippocampi of all animals. SEP amplitude was higher in both the PR (p < .005) and PO (p < .0005) groups compared to controls. This effect was found in all three components (P1, N1, P2) of the evoked response. Plasma nicardipine levels of 6-10 ng/ml were associated with mild hypotension.

We conclude that nicardipine improved neuronal function as measured by SEPs when administered before or after ischemia, most likely by interrupting the cytotoxic events occurring in cortical neurons during reperfusion.

THE PURPOSE OF THIS STUDY was to assess the effect of nicardipine, a dihydropyridine calcium entry blocker, on neuronal function in acute forebrain ischemia in rats. The drug was begun either prior to or immediately following ischemia and in both cases continued for 72 hours after the insult. The effect of this therapy was determined by measurement of cortical evoked potentials in treated and control animals.

Calcium blockers may be beneficial in cerebral ischemia because of two possible mechanisms: 1) relaxation of vascular smooth muscle and increase of cerebral blood flow (CBF), and 2) preservation of mitochondrial function in neurons by preventing the accumulation of intracellular calcium. It has been postulated that ischemia followed by reperfusion augments entry of this ion into neurons, and that calcium subsequently activates phospholipases producing a chain of events in the presence of oxygen resulting in the formation of damaging free radicals.

Pulsinelli's four vessel ischemia model is appropriate for the evaluation of the second mechanism. Severe delayed ischemic neuronal damage is produced in regions of brain which are immediately reperfused, CBF returning to normal by 24 hours so that a cytotoxic chain of events must be precipitated during the initial period of ischemia.

Somatosensory evoked potentials (SEP) have been...
used to assess neuronal function in animal models of cerebral ischemia.\textsuperscript{19, 20} Preliminary data from our laboratory in 8 non-operated non-ischemic rats established normal values for SEP amplitude and latency (fig. 1), and determined that daily measurements could be made under light anesthesia without affecting SEP amplitude or latency (Grotta J, Spydell J, Ostrow P [unpublished observations]).

**Methods**

Fifty three male Wistar rats weighing 250–300 gm (Hilltop Inc., Scottdale, PA) underwent successful surgery resulting in 20 minutes of forebrain ischemia. There were 28 ischemic control animals. Eight of these died during the 72 hours following ischemia leaving 20 for neuropathological and SEP measurements. Thirteen rats received nicardipine by continuous subcutaneous infusion starting 24 hours prior to ischemia and continuing for 72 hours of reperfusion (PR group). Two of these animals died, and one had seizures, leaving 10 for both neuropathological and SEP studies. Twelve rats began receiving nicardipine at the conclusion of the ischemic period and continuing throughout the 72 hour reperfusion period (PO group). Two of these animals died, leaving 10 for both neuropathological and SEP measurements.

The surgical technique and results of neuropathological and regional CBF studies in this model have been published.\textsuperscript{15-17} All animals were fed ad libetum throughout this experiment, and were weighed before surgery and 72 hours after ischemia. Each rat was anesthetized with an intramuscular injection of 0.3 cc of a premixed cocktail of Ketamine hydrochloride (100 mg/ml), Xylazine (20 mg/ml), and Acepromazine maleate (10 mg/ml) in a 3:3:1 ratio. Each rat then underwent bilateral vertebral artery cautery and cannulation of the jugular vein and tail artery according to the method of Pulsinelli.\textsuperscript{15} Each common carotid artery was isolated from its sympathetic nerve trunk and tagged with loose ligatures. In all animals a subcutaneous tunnel was formed in the dorsum for subsequent placement of an osmotic pump for drug administration. Blood pressure was monitored throughout the procedure by connecting the tail artery cannula to a Hewlett Packard transducer with strip chart recorder (Hewlett Packard Co., Palo Alto, CA). All rats were externally ventilated with a Harvard animal respirator (model #681, Harvard Bionics, South Natick, MA) and arterial blood gases measured on a standard blood analyzer (model 23A, Yellow Springs Instrument Co., OH). Core temperature was monitored and animals were placed under a heating lamp. Blood for analyses totalling 0.5 cc was obtained via the tail artery catheter.

Twenty four hours after the initial surgery, the animals were briefly exposed to ether. The common carotid arteries were delivered to the skin surface and occluded with aneurysm clips for 20 minutes, resulting in a loss of righting reflex (RR). Four rats out of an initial group of 57 regained RR during this period and were excluded leaving 53 for further analysis. Blood pressure, arterial blood gases, hematocrit, and serum glucose were measured at the time of carotid occlusion.

Nicardipine hydrochloride was dissolved in 3 ml sterile water with no preservatives. The mixture was added to an Alzet mini osmotic pump (model 2ML1, Alza Corp., Palo Alto, CA) in order to provide a continuous subcutaneous infusion of 0.05 mg/kg/hr for up to 96 hours according to the following calculation:

**Figure 1.** A. Representative normal somatosensory evoked potential in a sham operated non-ischemic rat. B. Amplitudes of P1, N1, and P2 waves and interwave latencies in 8 non-operated non-ischemic rats.
weight of animal (kg) \times 0.05 \text{ mg/kg/hr}
\frac{\text{predetermined pump delivery rate (ml/hr)}}{\text{# mg nicardipine added to 1 ml sterile water}} =

In all treated animals, sufficient nicardipine was added to the pump to infuse throughout the 72 hour reperfusion period. The pumps were filled under sterile conditions and then incubated in sterile saline for 4 hours at 37 degrees C before subcutaneous insertion. In the PR group, the pump was inserted at the time of vertebral artery cautery, 24 hours before ischemia. In the PO group, the pump was inserted at the end of the 20 minute ischemic period. No pump was placed into control animals. The surgical method was otherwise identical in PO, PR, and control groups and placement of the pump in the PO and PR animals did not necessitate additional anesthesia, surgical time or trauma. The volume of nicardipine plus sterile water infused was 10 \mu\text{L/hr}. This is a negligible volume and there was no difference in the subsequent state of hydration of treated and control animals.

Plasma nicardipine levels were obtained in PR and PO rats at variable intervals after placement of the pump, usually 4, 24, 48, 72, and 96 hours. In 10 rats, serial measurements were made during drug administration. Concentrations of nicardipine in samples of plasma were determined in the Department of Analytical and Metabolic Chemistry at Syntex Research, Palo Alto by means of a capillary column GC-ECD method which has a sensitivity of 1 ng of nicardipine per ml of plasma. 

Somatosensory evoked potentials (SEPs) and brainstem auditory evoked potentials (BEPs) were performed 72 hours after ischemia in 10 control, 10 PR, and 10 PO animals using a Grass model 10ERSB evoked potential instrument (Grass Instrument Co., Quincy, MA). Previous studies in our laboratory have shown changes in SEP amplitude as early as 24 hours following ischemia, but 72 hours was chosen for this study to correlate temporally with maximal neuropathological change. All calculations of SEP and BEP amplitude and latency were performed by an observer who was not aware of which treatment each rat had received. Rats were anesthetized with an intraperitoneal injection of chloral hydrate (0.5 mg/gm) prior to testing. Preliminary studies in our laboratory have shown that this anesthetic dose has no effect on either amplitude or latency of evoked potentials.

Cortical SEPs were recorded from a platinum subdermal electrode just anterior to the interaural line ("vertex" position). Preliminary studies in our laboratory have shown that a recording electrode at this position overlies the parietal cortex. Activity from this electrode was referenced to a distant subdermal electrode placed in the lumbar area, and a ground electrode was placed on a forelimb. Stimulating electrodes were inserted bilaterally in the perinasal area. Stimuli consisted of square wave electric pulses, 0.1 msec in duration at an intensity of 3.0 volts, delivered at a rate of 2 per second. Amplifier gain was adjusted to give a recognizable response (typically \times 2000), and highand low-pass filters of 30 and 3000 hz (50% down points, 9 db per octave roll-off) were used. A 20.48 msec sampling period was used and a total of 64 trials averaged to obtain the response. Responses were recorded so that vertex positivity produced a downward deflection. BEP responses were recorded between the previously mentioned vertex electrode and a subdermal electrode placed just anterior to the pinna of the stimulated ear. Stimuli were 0.1 msec, alternating polarity, square wave pulses at a rate of 11 per second delivered to a standard hearing aid transducer. A 3.5 cm length of polyethylene tubing was connected to the transducer and inserted into the external auditory canal of the animal. The intensity of the resulting click was approximately 60 db above the behavior threshold of a group of normal hearing adult, human subjects. Amplifier gain of \times 100,000 was used with high- and low-pass filters of 30 and 3000 hz respectively. A 10.24 msec sampling period was used and a total of 1024 trials averaged for each response. Responses were recorded so that vertex positivity produced an upward deflection.

Seventy-two hours after the ischemic insult while still under chloral hydrate anesthesia from EPs, 12 control, 10 PR, and 10 PO rats were infused with phosphate buffered 10% formalin and decapitated. Their brains were removed and mid-coronal slices 3 mm thick were embedded in paraffin, sectioned at 7 microns, and stained with hematoxylin and eosin.

Ischemic cell changes were quantitated in the hippocampus according to the percentage of cells with shrunken, eosinophilic cytoplasm and pyknotic nuclei. The thalamus and parietal cortex were also examined but our measurements focused on the hippocampus because of the severity of ischemic change seen and because the linear arrangement of its neurons facilitated quantitative observations. Care was taken to exclude "dark cell artifact" as described by Brown and Brierly. Severity of ischemic change was graded in linear subdivisions of the dentate and Ammon's horn regions as follows: 0–5% of cells involved; 6–25%; 26–50%; 51–75%; 76–100%. Grading was performed by an observer who was not aware of the treatment each rat had received. The results were plotted on diagrams of the sections to facilitate comparison. Rats were ranked by overall severity (proportion and linear extent) of ischemic damage. The Kruskal-Wallis test was performed and Spearmen Rho was calculated for control, PR, and PO groups.

Results

Physiologic parameters, except for mean arterial blood pressure (MABP), were comparable during ischemia in both groups of treated animals and controls (table 1). There was a modest but statistically significant (p < .05) reduction of MABP in PR animals. In the PO group, the drug was not started until after ischemia so MABP during ischemia was comparable to controls.

Mortality rate (# of deaths from onset of ischemia to
TABLE 1
Blood Pressure, PO2, pCO2, Glucose and Hematocrit (Hct) at Time of Ischemia in Control, PR, and PO Groups

<table>
<thead>
<tr>
<th></th>
<th>Stroke control</th>
<th>PR</th>
<th>PO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N = 20</td>
<td>N = 10</td>
<td>N = 10</td>
</tr>
<tr>
<td>MABP</td>
<td>105 ± 17</td>
<td>91 ± 12*</td>
<td>97 ± 18</td>
</tr>
<tr>
<td>PO2</td>
<td>108 ± 12</td>
<td>115 ± 12</td>
<td>114 ± 11</td>
</tr>
<tr>
<td>pCO2</td>
<td>29 ± 5</td>
<td>27 ± 4</td>
<td>31 ± 6</td>
</tr>
<tr>
<td>Glucose</td>
<td>115 ± 15</td>
<td>117 ± 17</td>
<td>108 ± 11</td>
</tr>
<tr>
<td>Hct</td>
<td>43 ± 3</td>
<td>42 ± 3</td>
<td>40 ± 4</td>
</tr>
</tbody>
</table>

(Means ± 1 standard deviation).
* p < .05.

72 hours after ischemia/total # animals undergoing ischemia was 29% (8/28) in the control group. There were fewer deaths in the PO and PR groups combined (16%, 4/25) but this difference was not statistically significant. Although behavioral parameters were not quantitated, motor skills including acquisition of food and water appeared better in treated animals. Nevertheless, all three groups demonstrated similar weight loss during the post ischemic period.

TABLE 2
Number of Brains in Each Treatment Group Demonstrating Hippocampal, Thalamic, and Parietal Cortex Ischemic Cellular Changes

<table>
<thead>
<tr>
<th></th>
<th>Control (N = 12)</th>
<th>PR (N = 10)</th>
<th>PO (N = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hippocampus</td>
<td>12</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Thalamus</td>
<td>6 (19)</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Parietal cortex</td>
<td>12 (38)</td>
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</tbody>
</table>

Ischemic cell changes were seen in the hippocampus, with the major impact in the dorsal part of Ammon's horn. When hippocampi were ranked for density and linear extent of cellular change, no significant difference in the ischemic insults produced was found between the 3 groups (fig. 2). Ischemic cellular changes were also seen in the thalami of 19% (6/32) and in the parietal cortex of 38% (12/32) (table 2). Differences between the treated groups and controls in the distribution of ischemia in hippocampus, thalamus, and parietal cortex were not significant, though the numbers of animals in the three groups were too small for statistically reliable subgroup analysis.

Nicardipine was found in the blood within 4 hours of the placement of the subcutaneous infusion pump. However, a predictable plasma level of 6–10 ng/ml was not reached until 20 hours (fig. 3). Variability in blood level occurred by 72 hours. The lowered MABP at the time of ischemia in the PR group occurred 24 hours following onset of therapy, at a time when the blood level averaged 6 ng/ml.

Figure 2. Distribution of ranking of hippocampal ischemic neuronal changes in control, PR, and PO groups. The rectangle excludes worst and best 25%, and horizontal line represents median rank for each group.

Figure 3. Plasma nicardipine levels during constant subcutaneous infusion (0.05 mg/kg/hr).
EFFECTS OF NICARDIPINE ON NEURONAL FUNCTION/Grotta et al

TABLE 3  
Amplitude of P1, N1, and P2 Waves in Control and Two Treatment Groups (micro volts)  

<table>
<thead>
<tr>
<th></th>
<th>P1</th>
<th></th>
<th>N1</th>
<th></th>
<th>P2</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>PR</td>
<td>PO</td>
<td>Control</td>
<td>PR</td>
<td>PO</td>
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<tr>
<td>0</td>
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<td>90</td>
<td>5</td>
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<tr>
<td>0</td>
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<td>30</td>
<td>15</td>
<td>30</td>
<td>30</td>
<td>200</td>
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<td>10</td>
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<td>75</td>
<td>20</td>
<td>10</td>
<td>75</td>
<td>30</td>
</tr>
<tr>
<td>0</td>
<td>40</td>
<td>30</td>
<td>100</td>
<td>40</td>
<td>35</td>
<td>5</td>
</tr>
<tr>
<td>0</td>
<td>40</td>
<td>10</td>
<td>35</td>
<td>135</td>
<td>125</td>
<td>310</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>30</td>
<td>0</td>
<td>0</td>
<td>438</td>
</tr>
<tr>
<td>0</td>
<td>135</td>
<td>35</td>
<td>125</td>
<td>30</td>
<td>100</td>
<td>80</td>
</tr>
<tr>
<td>0</td>
<td>10</td>
<td>540</td>
<td>25</td>
<td>0</td>
<td>100</td>
<td>75</td>
</tr>
<tr>
<td>0</td>
<td>450</td>
<td>1350</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>1350</td>
</tr>
</tbody>
</table>

Mean ± 1 SD  

<p>| | | | | | | | |</p>
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<tr>
<th></th>
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<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td>9 ± 20</td>
<td>80 ± 137</td>
<td>221 ± 429</td>
<td>46 ± 45</td>
<td>238 ± 236</td>
<td>272 ± 345*</td>
<td>104 ± 96</td>
<td>498 ± 436*</td>
</tr>
</tbody>
</table>

* = .05  
† = .025  

SEP amplitude 72 hours after ischemia was reduced in all three groups of animals compared to normal values. However, the mean amplitude of the N1 wave was significantly higher in both PR (p < .025) and PO (p = .05) animals, and the P2 wave was higher in PR (p < .025) animals compared to ischemic controls (table 3) (fig. 4).

Because the data were skewed and some measurements equalled zero, a log transform of the amplitude + 1 was calculated, and a repeated measures analysis of variance was performed, revealing a significant difference (p < .001) between the three treatment groups (ie control, PR, and PO) (table 4).

A series of one tailed t tests were performed on the transformed data to determine how the three treatment groups differed. Increased amplitude was found in the PR group (t = 3.1814, df = 58, p < .005) and the PO group (t = 4.0384, df = 58, p < .0005) compared to controls. No difference was found between the PR and PO groups (t = 0.7515, df = 58, N.S.). Higher amplitude was found in all three components of the SEP (ie the P1, N1, and P2 waves) in PR and PO animals (fig. 5).

Interwave latency did not differ from normal in either treatment group or ischemic controls nor was there a difference demonstrated between any of the 3 groups (table 5).

In all animals tested in all 3 groups of ischemic rats, BEPs remained normal at 72 hours when compared to previously established normal levels in our laboratory.

Discussion

The animal model used in this study causes ischemia and not infarction, and therefore is appropriate for evaluating therapy directed at preserving function in neurons which are threatened but presumably viable. We chose a 20 minute period of ischemia because it has been shown to produce severe but relatively selective damage to the hippocampus and neocortex. A shorter period of ischemia results in milder damage and therefore might not produce significant electrophysiologic changes for comparison between groups. A longer (ie 30 minute) period of ischemia causes higher mortality and also results in brainstem damage which we wanted to avoid. A recent negative report using verapamil in this model combined hypotension and 60–90 minutes of ischemia and may have produced too severe an insult to be ameliorated by calcium blockers.

Since BEPs remained normal in all animals, and since neuropathological abnormalities were most prominent in cortical neurons, we conclude that the subnormal SEP amplitudes in ischemic animals reflect cortical dysfunction, and that the differences in SEP amplitude between treated animals and controls reflect less severe impairment of cortical neuronal function in animals receiving nicardipine. This reasoning and pre-
Previously published studies indicate that SEP measurements can be used to assess cortical injury, but interaural recordings of SEPs cannot be localized with certainty to any particular region of cerebral cortex. Ischemic damage was seen in the parietal cortex underlying the recording electrode in 38% of ischemic animals. In addition, reduced SEP amplitude could be caused by damage to the thalamus (seen in another 19% of animals) or its connections to the cortex. Despite these uncertainties in the anatomical localization of our SEP recordings, it can be concluded that there was a lack of correlation between the amount of histologic change and cellular function since similar neuropathological damage occurred in the thalamus and hippocampal and parietal cortex in all groups despite significant differences in SEPs. While more detailed morphological examination by electron microscopy might detect a difference between treated and control animals, we conclude that light microscopic examination cannot detect differences in cellular injury which can result in differences in SEP amplitude. Another parameter of cortical function, learning ability, is also significantly disrupted in this model. Our results should be confirmed by further studies correlating learning ability with SEP measurements and pathological changes.

The mechanism by which nicardipine improves neuronal function was not determined with certainty by this study but the data suggest that the drug prevents damage to neurons occurring as a result of intra-neuronal calcium accumulation during the reperfusion period. In this model, CBF initially is low but hyperemia occurs immediately upon release of the carotid clips, and CBF is normal in most regions by 24 hours (fig. 6). Presumably, any therapy which improves cellular function in this model must either blunt the initial decrease in CBF or interrupt the cytotoxic chain of events triggered by ischemia and continuing during reperfusion. The increased SEP amplitude found in animals pretreated with nicardipine could be due to either mechanism, but the preservation of function occurring in the post-ischemia treated animals must be independent of any effect on CBF since blood levels of the drug were not predictably seen for up to 20 hours, when CBF in damaged areas was normal. The mechanism of action of this drug might be clarified by comparing CBF during and immediately following carotid clipping, and cerebral metabolic rate during reperfusion in treated and control animals.

A greater beneficial effect might have occurred in the PO animals if a more rapid or higher plasma drug level were achieved. The correct choice of dose in models of ischemia is hindered by the absence of any published measurement of drug levels in ischemic brain although a small amount of labelled nicardipine

### Table 4

<table>
<thead>
<tr>
<th>Source</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>81.74</td>
<td>89</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Between subjects</td>
<td>21.26</td>
<td>29</td>
<td></td>
<td>13.41</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>treatment</td>
<td>10.59</td>
<td>2</td>
<td>5.295</td>
<td></td>
<td></td>
</tr>
<tr>
<td>error between</td>
<td>10.67</td>
<td>27</td>
<td>0.395</td>
<td>25.82</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Within subjects</td>
<td>60.48</td>
<td>60</td>
<td></td>
<td>7.875</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>( P_1 ), ( N_1 ), ( P_2 )</td>
<td>15.75</td>
<td>2</td>
<td>7.875</td>
<td></td>
<td>&lt;.001</td>
</tr>
<tr>
<td>( P_1 ), ( N_1 ), ( P_2 \times ) treatment</td>
<td>28.28</td>
<td>4</td>
<td>7.070</td>
<td>23.18</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Error within</td>
<td>16.45</td>
<td>54</td>
<td>0.305</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

SS = sum of squares; df = degrees of freedom; MS = mean square; F = treatment/error; P = probability.

### Table 5

<table>
<thead>
<tr>
<th></th>
<th>P1-N1</th>
<th>N1-P2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (N = 10)</td>
<td>0.71 ± 1.75</td>
<td>0.97 ± 0.48</td>
</tr>
<tr>
<td>PR (N = 10)</td>
<td>0.62 ± 0.46</td>
<td>0.99 ± 0.49</td>
</tr>
<tr>
<td>PO (N = 10)</td>
<td>1.15 ± 1.20</td>
<td>0.79 ± 0.25</td>
</tr>
</tbody>
</table>

**Figure 5.** Distribution of the logarithm of \( P_1 \), \( N_1 \), and \( P_2 \) amplitude +1 in the control (c) and two treatment groups (PR, PO). The rectangle includes the mean ± one standard deviation.
has been demonstrated in the brain of normal animals after a single intravenous bolus. The dose chosen for this study was based on the few published animal studies of intravenous and oral administration of this drug. There are no published studies of subcutaneous administration but we chose this route because we wanted to maintain plasma levels of drug over the entire 72 hour period of reperfusion. While an initial intravenous bolus of the drug followed by subcutaneous infusion might achieve a more rapid plasma level, a plasma concentration of 6-10 ng/ml would be advantageous since hypotension occurred at this level.


**References**

The effect of nicardipine on neuronal function following ischemia.
J Grotta, J Spydell, C Pettigrew, P Ostrow and D Hunter

Stroke. 1986;17:213-219
doi: 10.1161/01.STR.17.2.213

Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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