Effects of naftidrofuryl oxalate on microsphere embolism-induced decrease in regional blood flow of rat brain

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1 The purpose of the present study was to determine whether naftidrofuryl oxalate (naftidrofuryl), a vasodilator, is capable of improving brain regional blood flow of animals in sustained ischaemia.

2 Cerebral ischaemia was induced by injecting 900 microspheres (48 μ m in diameter) into the right internal carotid artery of rats. Cerebral blood flow of brain regions was measured by a hydrogen clearance method on the 3rd, 7th and 28th days after the onset of ischaemia. Ischaemic animals were treated with naftidrofuryl, 15 mg kg⁻¹ day⁻¹ i.p., from the first to 28th day.

3 Microsphere-embolism caused a sustained decrease in cortical and striatal blood flow over a period of 28 days, whereas hippocampal blood flow was decreased on the 3rd day but not on the 7th or 28th day. On the 3rd day, the striatal and hippocampal but not cortical blood flow of naftidrofuryl-treated, microsphere-embolized rats was higher than untreated rats. On the 7th and 28th days, the cortical and striatal blood flow of the treated and untreated animals did not differ.

4 Brain slices from microsphere-embolized rats contained areas, which were not stained with triphenyltetrazolium chloride (TTC), to a similar degree on the 3rd, 7th and 28th days, indicating the genesis of cerebral infarction. TTC-unstained areas of microsphere-embolized rats that had received naftidrofuryl treatment were smaller than those of untreated rats on the 3rd and 7th days, but not on the 28th day.

5 The results suggest that naftidrofuryl improves cerebral circulation impaired by microsphere-induced ischaemia and this higher level of cerebral blood flow of the treated animal may account for the delayed development of cerebral infarction.

Keywords: Cerebral blood flow; cerebral cortex; hippocampus; microsphere-embolism; naftidrofuryl; striatum

Introduction

Naftidrofuryl oxalate, [2-(diethylamino)ethyltetrahydro- α -(1-naphthylmethyl)-2-furanpropionate ester oxalate], (naftidrofuryl), is a cerebral and peripheral vasodilator (Admani, 1978), and has been found to increase cerebral blood flow in anaesthetized animals (Hagiwara *et al.*, 1973; Young *et al.*, 1983; Hiramatsu *et al.*, 1988) and man (Ohtomo *et al.*, 1986). However, the mechanism by which this agent induces its beneficial effect on brain function under pathophysiological conditions is poorly understood. In the present study we attempted to determine whether naftidrofuryl oxalate increased the blood flow of brain regions subjected to sustained ischaemia. For this purpose, we induced cerebral ischaemia by microsphere-embolism. Cerebral embolism with microspheres has been shown to induce biochemical, electrophysiological, neurological and morphological abnormalities (Siegel *et al.*, 1972; Kogure *et al.*, 1974; Bralet *et al.*, 1979; Sugi *et al.*, 1984; Kiyota *et al.*, 1985; Narumi *et al.*, 1986). The microspheres (diameters 15–50 μ m) are considered to produce widespread pre-capillary occlusion, resulting in a multi-focal, long-lasting cerebral ischaemia and infarction in the brain. Using this ischaemic model, we examined the effects of naftidrofuryl on the cerebral blood flow of a number of brain regions, cerebral cortex, striatum and hippocampus, which are vulnerable to ischaemia (Kirino, 1982; Pulsinelli *et al.*, 1982; Smith *et al.*, 1984).

Methods

Male Wistar rats weighing 200–220 g (Charles River Japan Inc., Atsugi, Japan) were used in the present study. The animals were maintained under artificial conditions at 23 $\pm$ 1°C, with a constant humidity of 55 $\pm$ 5%, a cycle of 12 h of light and 12 h of dark and free access to food and tap

water, according to the Guidelines of Experimental Animal Care issued by the Prime Minister's Office of Japan. Microsphere-induced cerebral embolism was conducted by the methods previously described (Miyake *et al.*, 1989). Briefly, rats were anaesthetized with sodium pentobarbitone, 35 mg kg⁻¹ i.p. After ligation of the right external carotid and pterygopalatine arteries, 900 microspheres (47.5 $\pm$ 0.5 μ m in diameter; NEN-005, New England Nuclear Inc., Boston, Mass, U.S.A.), suspended in 20% dextran solution, were injected into the right internal carotid artery through a polyethylene catheter (3 French size, 1.0 mm in diameter, Atom Co., Tokyo, Japan) inserted into the right common carotid artery. Rats that underwent sham operation were injected with the same volume of vehicle without microspheres, and their right common carotid arteries were ligated. The control group comprised non-operated rats and were used to examine normal levels of haemodynamic and histological variables of the intact animal. Fifteen hours after the operation, the animal behaviour was scored on the basis of paucity of movement, truncal curvature and forced circling during locomotion, which are considered to be typical symptoms of stroke in rats (Furlow & Bass, 1976; McGraw, 1977). Each symptom was scored from 3 to 0, 3 very severe, 2 severe, 1 moderately severe, 0 little or no symptoms. The sum of these scores was evaluated as a marker of neurological deficits. Rats with 7–9 points were considered type A; 4–6 points, type B; and 3–0 points, type C. The type A animals were used in the following experiment. The animal's behaviour was examined at 09 h 00 min every day throughout the experiment.

Treatment of microsphere-injected and sham-operated rats with naftidrofuryl

After ensuring their stroke-like symptoms, the microsphere-injected and sham-operated rats were treated with naftidro-

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furyl 15 mg kg⁻¹ day⁻¹ i.p., from the first to the 28th day after the operation. The dose used in the present study was shown to be effective in restoring biochemical parameters of microsphere-injected rats in previous studies (Miyake *et al.*, 1989; Takeo *et al.*, 1991).

Measurements of cerebral blood flow

Cerebral blood flow was measured in control, microsphere-injected and sham-operated rats with and without naftidrofuryl-treatment. The animals were anaesthetized with intraperitoneal administration of sodium pentobarbitone 35 mg kg⁻¹. Arterial blood pressure was monitored via a cannula inserted into the right femoral artery and measured by means of a pressure transducer (model AP-601G, Nihonkohden Co., Tokyo, Japan) throughout the experiment. The heart rate measurement was triggered through the ECG (lead II) and counted by means of a heart rate counter (model AT-601G, Nihonkohden Co., Tokyo, Japan) throughout the experiment. The rectal temperature of the rat was maintained at 36.5 ± 0.5°C by warming the animal with an electronic panel-heater placed underneath it. Tissue blood flow of brain regions was measured by hydrogen clearance using a hydrogen clearance flow meter (model UPS-400, Unique Medical Inc., Tokyo, Japan). The head of the rat was fixed in a head holder (model SR-5, Narishige Instrument Co., Tokyo, Japan). Three small bar holes were made with an electric mini-drill (model 28400, Proxxton, Germany) in the skull of each side. Two Teflon-coated platinum electrodes, 100 µm in diameter, with a 1-mm uncoated portion at their tips and plated with black platinum, were positioned at a depth of 2 mm, 2 mm posterior and 2 mm lateral to the bregma for measurement of blood flow of the cortex, at a depth of 4 mm, 2 mm anterior and 3 mm lateral to the bregma for that of the striatum, and at a depth of 5 mm, 6 mm posterior and 5 mm lateral to the bregma for that of the hippocampus of each hemisphere according to the *Atlas of Anatomy of Rat Brain* (Paxinos & Watson, 1986), using a stereotactic apparatus (model SR-5, Narishige Instrument Co., Tokyo, Japan). The reference Ag-AgCl electrode was inserted under the skin of the neck. The animals inhaled a gas mixture of 90 ml min⁻¹ air and 10 ml min⁻¹ hydrogen through a mask loosely fitted to their nose. After ensuring the equilibration of the brain tissue with hydrogen gas, the cerebral blood flow was measured. At about 30 min intervals, the blood flow of the other brain regions was similarly determined.

In a separate set of experiments, blood gas analysis of control and sham-operated and microsphere-injected rats were performed on the 3rd day after embolism under the present experimental conditions to ensure the validity of these experimental conditions. The animals were anaesthetized with sodium pentobarbitone, 35 mg kg⁻¹, as described above. A cannula was inserted into the right femoral artery for blood sampling. The animals inhaled air containing 10% hydrogen gas as described above. Blood (0.2 ml) was sampled before inhalation (0 min), and three times after equilibration of hydrogen gas in the tissue (30, 60 and 90 min). The blood was immediately analysed with a blood gas analyzer (model 288, Ciba-Corning Japan, Tokyo, Japan).

Measurement of infarct areas

To determine the infarct area, microsphere-injected and sham-operated animals with and without naftidrofuryl-treatment were lightly anaesthetized with ether and decapitated at different time intervals (the 3rd, 7th and 28th days) after the operation. Rats that did not undergo any operation (controls) were also decapitated. The brains were rapidly isolated; after cooling in a stainless-steel container surrounded with ice, the brain was positioned on a brain holder and coronally sectioned with razors, 3, 5, and 7 mm from the frontal pole. The sections of brain tissue were

incubated at 37°C for 30 min with 2% of 2,3,5-triphenyl-tetrazolium chloride (TTC) in physiological saline (Benderson *et al.*, 1986). The slices were briefly immersed in a 10% formalin solution and then photographed. The sum of TTC-stained, and TTC-unstained (including weakly stained) areas of three brain slices were estimated by a planimetric method.

Statistics

The results are expressed as mean ± s.e.mean. Statistical significance between drug-treated and untreated groups was evaluated by Student's *t* test. Analysis of variance followed by Dunnett's *t* test was employed for evaluation of significance of the time course of changes in variables. The Wilcoxon rank sum test was used for comparison of changes in behavioural scores with and without naftidrofuryl oxalate treatment. A confidence value of >95% was considered significant (*P* < 0.05).

Results

The animals (200 rats) were inspected 15 h after surgery. Forty-two rats (21%) died within 24 h of the embolism. One hundred and thirty embolized rats (65%) showed type A symptoms, 20 rats (10%) type B, and 8 rats (4%) type C, which was similar to previous results (Miyake *et al.*, 1989; 1993; Takeo *et al.*, 1991). Among the type A rats, 20 rats died within 3 days of the embolism. Consequently, 110 type A rats were used in the following experiment. No neurological deficits were seen in 104 sham-operated rats.

In the first set of experiments, we performed blood gas analysis to ascertain the physiological profile of control, sham-operated and microsphere-injected rats under the same conditions as those undergoing determination of cerebral blood flow according to the hydrogen gas clearance method. The microsphere-injected and sham-operated rats on the 3rd day after the operation were chosen as typical samples in this series of experiment. As shown in Table 1, there were no differences in pH, *P*O₂ and *P*CO₂ values of the control, sham-operated and microsphere-injected rats during the entire experimental period (0–90 min).

Mean arterial blood pressure and heart rate of microsphere-injected and sham-operated rats were routinely monitored before and during measurements of cerebral blood flow at different time periods after microsphere-embolism. Table 2 shows mean arterial pressure and heart rate of sham-

Table 1 Blood gas analysis of rats under the same conditions as those for measurements of regional cerebral blood flow

	Time after the start of experiment			
	0	30	60	90 min
<i>Control</i>				
pH	7.37 ± 0.01	7.40 ± 0.01	7.39 ± 0.01	7.40 ± 0.01
<i>P</i> O ₂	90.7 ± 2.3	93.6 ± 2.4	96.5 ± 3.0	97.7 ± 2.7
<i>P</i> CO ₂	40.1 ± 1.5	40.0 ± 1.2	40.5 ± 0.8	38.2 ± 1.3
<i>Sham-operated</i>				
pH	7.44 ± 0.01	7.49 ± 0.03	7.44 ± 0.03	7.46 ± 0.02
<i>P</i> O ₂	95.6 ± 2.3	95.4 ± 2.4	94.3 ± 1.5	97.1 ± 1.3
<i>P</i> CO ₂	40.1 ± 2.1	41.0 ± 1.7	40.2 ± 4.0	37.5 ± 2.7
<i>Microsphere-injected</i>				
pH	7.40 ± 0.01	7.43 ± 0.01	7.42 ± 0.02	7.45 ± 0.01
<i>P</i> O ₂	94.2 ± 1.5	94.3 ± 0.9	94.9 ± 1.2	95.3 ± 1.5
<i>P</i> CO ₂	40.7 ± 1.0	40.8 ± 0.4	41.3 ± 1.4	41.3 ± 0.7

Blood samples were taken from animals anaesthetized with sodium pentobarbitone. Values for *P*O₂ and *P*CO₂ are expressed as mmHg. Each value represents the mean ± s.e.mean of 6 (control), 4 (sham-operated) and 4 (microsphere-injected) experiments.

Table 2 Mean arterial blood pressure and heart rate of microsphere-injected and sham-operated rats with and without naftidrofuryl oxalate treatment

	Mean arterial pressure (mmHg)				Heart rate (beats min ⁻¹)			
	Time after the start of experiment	Time after the start of experiment	Time after the start of experiment	Time after the start of experiment	Time after the start of experiment	Time after the start of experiment	Time after the start of experiment	Time after the start of experiment
	0	30	60	90 min	0	30	60	90 min
Control	103 ± 2	102 ± 2	104 ± 2	105 ± 2	421 ± 17	422 ± 10	425 ± 9	420 ± 11
Sham-operated								
Untreated	100 ± 4	106 ± 4	110 ± 5	111 ± 6	412 ± 17	419 ± 13	425 ± 14	433 ± 12
Treated	105 ± 8	108 ± 7	107 ± 7	105 ± 3	436 ± 26	437 ± 29	462 ± 29	425 ± 6
Microsphere-injected								
Untreated	105 ± 2	106 ± 5	107 ± 2	101 ± 5	411 ± 13	407 ± 15	403 ± 12	422 ± 11
Treated	103 ± 3	101 ± 5	105 ± 6	103 ± 5	407 ± 7	408 ± 9	418 ± 10	411 ± 22

Measurements of blood pressure and heart rate were conducted in rats under sodium pentobarbitone anaesthesia. Values are shown only in sham-operated and microsphere-injected rats at the 3rd day after the operation. Mean arterial blood pressure and heart rate were similar in the operated and sham-operated rats irrespective of time and treatment. Each value represents the mean ± s.e. mean of 6 to 10 experiments.

operated and microsphere-injected rats on the 3rd day after the operation with and without naftidrofuryl oxalate treatment. There were no significant differences in either parameter between microsphere-injected and sham-operated rats with and without naftidrofuryl oxalate treatment on any day after the operation. Likewise, no appreciable changes were observed in either parameter of microsphere-injected animals throughout the measurements of regional blood flow. Similarly, there were no changes in mean arterial blood pressure and heart rate of microsphere-embolized and sham-operated animals examined on the 7th and 28th days after the operation.

The effects of naftidrofuryl on cortical, striatal and hippocampal blood flow of the right hemispheres of microsphere-injected, and sham-operated rats are shown in Figure 1. On the 3rd day after the operation, cortical, striatal and hippocampal blood flow was significantly decreased after microsphere-embolism. Treatment with naftidrofuryl significantly attenuated the decrease in blood flow of striatal and hippocampal, but not cortical, regions of the right hemisphere. On the 7th and 28th days after the operation, cortical and striatal blood flow was still lower than control. Naftidrofuryl did not attenuate the decrease in blood flow of cortical and striatal regions on the 7th and 28th days. In sham-operated animals, there were no significant reductions in the blood flow of these brain regions of the right hemisphere on the 3rd, 7th and 28th days after the operation. Likewise, naftidrofuryl had no significant effect on the blood flow of the three brain regions of sham-operated rats throughout the experiment. In the left hemisphere, there were no significant alterations in the blood flow of these brain regions at any period of the experiment examined.

The brains of microsphere-injected and sham-operated rats were isolated on the 3rd, 7th and 28th days after the operation and used for gross observation and TTC-staining. On the 3rd day, cerebral vasodilatation, oedema, and haemorrhage were seen in the right hemisphere of microsphere-injected rats. There was a marked degeneration and/or atrophy of the right hemisphere of microsphere-injected rats on the 28th day after the operation. No such abnormalities were seen in the left hemisphere of microsphere-injected rats or either hemisphere of sham-operated rats throughout the experiment.

TTC-staining of brain slices was performed to determine cerebral infarct area (Figure 2). The TTC-unstained areas (including weakly stained areas) of the right hemisphere of microsphere-injected rats on the 3rd day were approximately 79% of the corresponding sham-operated animals. The TTC-stained areas of the right hemisphere on the 7th and 28th days were almost the same as those on the 3rd day. Treatment with naftidrofuryl resulted in a significant attenuation of the development of TTC-unstained area on the 3rd and

Figure 1 The effects of naftidrofuryl oxalate treatment on blood flow of the cerebral cortex, striatum and hippocampus of the right hemisphere of microsphere-injected (M and M+Naf), sham-operated (S and S+Naf) rats with (+Naf) and without naftidrofuryl oxalate treatment, and control rats (C), respectively. Measurement of cerebral blood flow was carried out under sodium pentobarbitone anaesthesia. Control values of cortical, striatal and hippocampal blood flow of the right hemisphere were 42.9 ± 4.7 , 36.4 ± 1.3 and 54.6 ± 3.1 ml min⁻¹ 100 g⁻¹ tissue, and those of the left hemisphere 44.4 ± 4.3 , 38.1 ± 1.8 and 54.3 ± 3.2 ml min⁻¹ 100 g⁻¹ tissue, respectively ($n = 6$). Each value represents the mean ± s.e. mean of 6 (control), and 8 to 10 (sham and microsphere embolism) experiments. There were no significant alterations in cerebral blood flow of the left hemisphere of microsphere-injected rats on the 3rd, 7th and 28th days after the operation regardless of treatment with or without naftidrofuryl. *Significantly different from the corresponding sham-operated group and †significantly different from microsphere-injected group ($P < 0.05$).

7th days, but not 28th day, after the operation. No TTC-unstained area was seen in the brain slices of either hemisphere of sham-operated rats, even though the right common carotid arteries of all animals were permanently ligated.

Figure 2 The sum of triphenyltetrazolium chloride (TTC)-stained areas of three brain slices from the right hemisphere of microsphere-injected rats (M and M+Naf) and those of sham-operated rats (S and S+Naf) with (+Naf) and without naftidrofuryl treatment on the 3rd, 7th and 28th days after the operation. Values are expressed as the mean $\pm$ s.e.mean of 6 (control) and 8 to 9 (sham and microsphere-embolism) experiments. The TTC-stained areas of the right and left hemispheres of control animals were 214.5 ± 0.9 and 214.1 ± 0.3 mm², respectively ($n = 6$). There were no significant alterations in the TTC-stained areas of the left hemisphere of microsphere-injected and sham-operated rats regardless of treatment with or without naftidrofuryl. *Significantly different from the sham-operated groups and †significantly different from the untreated group ($P < 0.05$).

Animal behaviour was examined in rats employed for the studies on cerebral blood flow and TTC staining areas throughout the experiments; the initial numbers of the untreated and treated animals were 52 and 54, respectively. The behavioural scores of untreated, microsphere-injected animals on the 1st, 3rd, 7th and 28th days after the operation were 7.6 ± 0.4 , 6.7 ± 0.6 , 2.8 ± 0.6 and 0.3 ± 0.3 , while those of naftidrofuryl oxalate-treated, microsphere-injected animals were 7.6 ± 0.2 , 5.6 ± 0.5 , 2.6 ± 0.4 and 0.2 ± 0.2 , respectively. Although a slight improvement of the behavioural scores was observed in the treated animals from the 3rd and 7th day after the operation, there was no significant difference in the scores between the treated and untreated animals throughout the experiments.

Discussion

In the present study we observed variable changes in blood flow of the brain regions: that is, a sustained decrease in cortical and striatal blood flow, and a decrease in hippocampal blood flow which was reversed 7 days after the embolism. The findings provide direct evidence that microsphere-embolism induced sustained ischaemia or oligaemia in some brain regions of the cerebral hemisphere into which microspheres were introduced. Variable reversal of blood flow of brain regions has also been documented in rats with middle cerebral artery occlusion; relatively better recovery of blood flow in the anterior cingulate gyrus and hippocampal regions and very little recovery of blood flow in the frontal cortical and dorsal caudate regions, 48 h after the occlusion (Hakim *et al.*, 1992).

Reduction in blood flow of brain regions after microsphere-embolism can be predicted from the findings of our previous studies. These included decreases in tissue high-energy phosphates (Takeo *et al.*, 1992) and acetylcholine (Taguchi *et al.*, 1993), and increases in tissue glucose and lactate (Takeo *et al.*, 1991; 1992). These changes are consistent with an ischaemic state (Siesjö *et al.*, 1976; Welsh *et al.*, 1977; Gibson *et al.*, 1981). From both circulatory and biochemical observations, it could be concluded that microsphere-embolism induces a sustained decrease in cerebral

blood flow of the cerebral cortex and striatum, which results in a profound metabolic disturbance in these regions.

We also observed variable effects of naftidrofuryl on the decreased levels of blood flow of the brain regions. That is, the decrease in striatal blood flow was partially, but significantly, attenuated by treatment with naftidrofuryl.

Furthermore, the decrease in hippocampal blood flow was attenuated almost completely by treatment with naftidrofuryl, whereas treatment did not restore the decreased cortical blood flow on the 3rd day. Treatment with naftidrofuryl did not result in a significant recovery of the blood flow of these brain regions thereafter. The results suggest that these cortical regions are severely damaged following microsphere-embolism and naftidrofuryl treatment is capable of improving cerebral blood flow of certain brain regions, which are damaged to a relatively lesser degree, but only at an early stage (3 day) of cerebral ischaemia. This implies that naftidrofuryl substantially retards, but may not prevent, the development of ischaemic damage to brain regions.

The TTC-staining method is a convenient method of determining cerebral infarct area and a close correlation with evaluation of infarct size by haematoxylin-eosin has been described in the literature (Benderson *et al.*, 1986; Isayama *et al.*, 1991). Our results show the formation of widespread TTC-unstained or weakly stained areas in the brain slices of the right hemisphere from microsphere-injected animals from 3 to 28 days after the operation, which demonstrated the development of cerebral infarction in the brain. In accordance with this, in a previous microscopic study (Miyake *et al.*, 1993), we observed areas sparsely stained with haematoxylin-eosin in the right hemisphere, which suggests a widespread degeneration of the microsphere-injected hemisphere. Necrotic areas, variable in size and shape, with small haemorrhagic foci were also widely observed in various brain regions. This is indicative of multi-focal necrosis. These morphological abnormalities were partially reduced by treatment with naftidrofuryl (Taguchi *et al.*, 1994). This is in good agreement with our present findings of the delayed development of TTC-unstained areas in the microsphere-embolized, naftidrofuryl-treated rat brain slices. In contrast, there are discrepancies in the results regarding the effects of the agent on the development of infarct areas and cerebral blood flow in the present study. That is, the effects of naftidrofuryl on the cerebral blood flow were seen only on the 3rd day and the effects on the TTC-unstained areas were observed both on the 3rd and 7th days after the operation. It is frequently observed in an ischaemic animal that biochemical alterations precede histological alterations. Actually, in this microsphere-embolized model, metabolic abnormalities in the ipsilateral brain hemisphere were observed immediately or one day after the microsphere-embolism, whereas TTC-unstained areas were poorly developed 12–24 h after the embolism (Takeo *et al.*, 1992; Miyake *et al.*, 1992; Taguchi *et al.*, 1993). Thus, it is conceivable that the influence of naftidrofuryl-induced improvement of cerebral circulation may retard the development of morphological damage for a few days.

In the present study, the behavioural scores of the microsphere-embolized animal which are considered to be stroke-like symptoms in rats (Furlow & Bass, 1976; McGraw, 1977) improved only to a limited degree, despite improvement of cerebral blood flow of the right hemisphere of microsphere-embolized rats 3 days after the operation by treatment with naftidrofuryl. One could not argue, however, that the two results are inconsistent because of uncertainty concerning the precise area that controls animal behaviour and the extent of restoration of cerebral blood flow necessary for improvement of animal behaviour. It would also be unreasonable to correlate directly alterations of behavioural score, a qualitative parameter, with changes in cerebral blood flow, a quantitative parameter.

In summary, we found that naftidrofuryl improved striatal and hippocampal blood flow at an early stage of cerebral ischaemia following microsphere-embolism. This was assoc-

iated with a delayed development of cerebral infarction as shown by TTC-staining. This improvement in cerebral blood flow may play a role in the naftidrofuryl-induced improvement of metabolic disturbances, such as depletion of tissue high-energy phosphates (Takeo *et al.*, 1991), decreases in acetylcholine and neurotransmitter amino acids (Taguchi *et*

al., 1994), and increases in tissue glucose and lactate (Takeo *et al.*, 1991).

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