Neurol Med Chir (*Tokyo*) *41, 107～114, 2001*

Neuroprotection by Intrathecal Application of Liposome-entrapped Fasudil in a Rat Model of Ischemia

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Abstract

Pharmacological treatment for cerebral ischemia cannot attain sufficiently high concentrations of the drugs in the cerebrospinal fluid (CSF) without precipitating systemic side effects. The objective of this study is the development of a liposomal drug delivery system that maintains effective concentrations of protein kinase inhibitors fasudil in the CSF, resulting in neuroprotection against cerebral ischemia. Focal cerebral ischemia in rats was induced by middle cerebral artery occlusion using an intraluminal suture technique. Treated rats received 0.25 mg liposome-entrapped fasudil via the cisterna magna 2 hours after ischemic insult. Control rats received drug-free liposomes. Neurological condition and the infarct size were assessed at 24 and 72 hours after ischemia. The concentration of liposome-entrapped fasudil in the CSF was measured before sacrifice. Treated animals showed significantly improved neurological outcomes after the 24-hour observation period compared to the control group ($p < 0.001$). Treatment with 0.25 mg liposomal fasudil resulted in a reduction in the infarct area (24 hours: 29.0 ± 4.4%, 72 hours: 28.1 ± 3.9% of total brain slices) compared to controls (49.6 ± 4.6%, $p < 0.001$), but there was no statistical difference between 24 and 72 hours. At 24 hours post-administration, CSF concentrations of liposome-entrapped fasudil were 45.4 ± 31.5 μg/ml (20% of the injected dose). A single intrathecal injection of liposomal fasudil can maintain a therapeutic drug concentration in the CSF over a period of time, significantly decreasing infarct size in a rat model of acute ischemia.

Key words: cerebral ischemia, drug delivery systems, liposomes, protein kinase inhibitor, fasudil

Introduction

Fasudil hydrochloride (1-5-isoquinolinesulphonyl-homopiperazine) has shown neuroprotective properties against ischemia-induced neuronal damage in rats and gerbils, but high doses of fasudil (30 mg/kg) are required to reduce infarct areas using intraperitoneal administration.[22] The peripheral route of administration requires larger doses of drugs than the intrathecal route, which could lead to adverse effects, reducing the therapeutic efficacy of the drug. The intrathecal route can overcome the inability of peripherally administered calcium antagonists to penetrate the blood-brain barrier and to allow diffuse distribution of drugs through the entire neuraxis, and is effective for drugs such as nimodipine.[13,26] However, the morbidity associated with long-term intrathecal drug infusion through indwelling catheters, which are required for continuous drug administration, has precluded the application of this procedure in the clinical setting. In addition, assuming bolus administration by the intrathecal route was feasible, the therapeutic window for the free drug may be too short. Therefore, we have attempted to address this issue by developing a sustained-release form of the drug that could be implanted intracranially at the time of surgery or injected by lumbar puncture.

The objective of this study is to seek 'proof of principle' for the hypothesis that a sustained release liposomal drug delivery system will maintain an effective concentration of fasudil in the cerebrospinal fluid (CSF) and exert a neuroprotective effect against cerebral ischemia in a rat model.

Received July 21, 2000; Accepted November 27, 2000

Materials and Methods

I. Preparation of liposomes

Liposomes were prepared according to the method of Allen and Hansen.[3] Liposomes were composed of hydrogenated soy phosphatidylcholine:cholesterol (HSPC:CHOL) at 2:1 molar ratio. Fasudil-loaded liposomes were formed by drug encapsulation by remote loading using an ammonium sulfate gradient.[10] Dried lipid films were hydrated in 250 mM ammonium sulfate (pH 3.0). To produce homogeneously-sized liposome preparations, the liposomes were sequentially extruded (Lipex Biomembranes Extruder, Vancouver, British Columbia, Canada) through a series of polycarbonate filters (Nuclepore Corp., Pleasanton, Calif., U.S.A.) with pore sizes ranging from 0.4 to 0.08 μm. The mean diameter of liposomes was determined by dynamic light scattering using a Brookhaven B190 submicron particle size analyzer (Brookhaven Instruments Corp., Holtsville, N.Y., U.S.A.) and was in the range of 110 $\pm$ 10 nm. The external buffer was then exchanged by eluting through a Sephadex G50 column equilibrated with 10% sucrose (pH 8.0). Fasudil (Sigma Chemical Co., St. Louis, Mo., U.S.A.) was remote-loaded into the liposomes by adding it to the extruded liposomes at a phospholipid:fasudil ratio of 1:0.4 (w/w) and incubating for 1 hour at 65°C. Liposome-entrapped fasudil was separated from free fasudil using a Sephadex G50 column eluted with 25 mM N-2-hydroxyethyl piperazine-N'-2-ethansulfonic acid and 140 mM NaCl buffer (pH 7.4). The concentration of the liposome-entrapped fasudil was determined by spectrophotometry (λ = 320 nm), and phospholipid concentrations were determined using the Bartlett colorimetric assay.[7] The loading efficiency of fasudil into liposomes was then calculated.

II. In vitro drug-release kinetics

The in vitro drug-release kinetics of the liposome-entrapped fasudil was measured in control CSF (Sigma Chemical Co.). Liposomes (0.5 ml) containing 0.25 mg of fasudil and 0.76 μmol phospholipid were incubated in 4.5 ml of CSF at 37°C for 48 hours. Periodically, 0.5 ml of the medium was withdrawn and replaced with an equivalent volume of fresh CSF to mimic CSF turnover in vivo. Free fasudil was separated from liposome-entrapped fasudil using a Sephadex G50 column, as above. Following dissolution in 100% methanol, the concentration of the fasudil in liposomes was then determined by spectrophotometry (λ = 320 nm), and phospholipid concentrations were determined using the Bartlett colorimetric assay.[7]

III. Acute toxicity of fasudil following intrathecal administration

Five male Sprague-Dawley rats weighing 300–400 g were used to investigate the acute adverse effects of free (non-entrapped) fasudil by intrathecal administration. Each rat received a different dose of fasudil (0.01 to 0.25 mg) via the cisterna magna. After administration of the drug, the rats were carefully monitored for any respiratory disturbances or convulsions. The experimental protocol adhered to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health Publication 86–23, revised 1985).

IV. Experimental model of cerebral ischemia

Thirty male Sprague-Dawley rats weighing 300–400 g were used for the experiments. Animals were housed in the vivarium with free access to water and food in a 12-hour, day-night cycle. Approval from the institutional ethics committee (University of Alberta) was obtained for all procedures.

Anesthesia was induced with 2.0% halothane in a 1:2 mixture of O_2/N_2O and was maintained with a mixture of 70% N_2O, 30% O_2, and 1.0% to 1.5% halothane using a face mask. Rectal temperature was maintained at approximately 37 $\pm$ 0.5°C throughout the surgical procedure using a heating pad and an overhead lamp. A catheter was inserted into the femoral artery for blood sampling and continuous monitoring of blood pressure. The left middle cerebral artery (MCA) was occluded using the endovascular suture technique.[16,21] In brief, after the left cervical carotid bifurcation was exposed through a midline incision, the distal internal carotid artery (ICA) was carefully dissected free from the adjacent vagus, hypoglossal, and glossopharyngeal nerves. A microvascular clip was placed on the pterygopalatine artery, which was the first branch of the ICA beneath the digastric muscle. The left external carotid artery (ECA) was electrocauterized and cut distal to the superior thyroid artery, and subsequently a microvascular clip was placed at the origin of the ECA. A 3-0 monofilament nylon thread was introduced into the ECA lumen after removal of the ECA clip, and both the ECA stump and the nylon thread were sealed with a 6-0 silk thread. The nylon thread was advanced approximately 18 mm from the origin of the ECA, so that the proximal segment of the anterior cerebral artery (ACA) and the origin of the MCA were occluded by the tip of the nylon thread. Finally, the incision was sutured. The animals were allowed to recover from anesthesia and were given free access to food and water. None of the rats showed signs of distress or

pain during the postoperative period.

Thirty rats were randomly assigned to either a control group or a treated group in a blinded manner. The animals were again anesthetized 2 hours after the ischemic insult for intrathecal administration of the liposomal fasudil or the drug-free liposomes. The craniovertebral junction was exposed using a midline incision. Then, either 0.25 mg liposomal fasudil (treated group, n = 20) or drug-free liposomes (control group, n = 10) was applied to the cisterna magna with a 27 gauge needle. The drug-free liposomes contained the same amount of phospholipid as the fasudil-loaded liposomes. After the procedures, the wound was stitched together and the animals were treated as mentioned above.

V. Neurological deficit evaluation

Neurological deficit evaluation was conducted in all 30 rats at 4, 24, and 72 hours after ischemic insult as a blinded manner.[9] The neurological findings were scored in three successive trials on a four-point grade-scale: no observable deficit (0); forelimb flexion (1); forelimb flexion and decreased resistance to lateral push (2); and forelimb flexion, decreased resistance to lateral push, and unilateral circling (3). The animals were closely observed for any other neurological abnormalities not included in the grade-scale evaluation.

VI. Evaluation of infarct damage

The infarct brain tissue in the left MCA territory was differentiated using the 2,3,5-triphenyltetrazolium chloride (TTC) staining method.[8] Twenty-four and 72 hours after ischemic insult, the rats were again anesthetized with halothane and then decapitated. The fresh brains were sectioned coronally into 2-mm slices, after which the sliced brains were immersed at 37°C for 30 minutes in a 1% solution of TTC (Sigma Chemical Co.) in normal saline and stored in 10% phosphate-buffered formalin for fixing. The areas of cerebral infarction and both hemispheres on each slice were analyzed using an image processing software-program, Adobe Photoshop 5.0 (Adobe Systems Inc., San Jose, Calif., U.S.A.). Measurements by an investigator (Y.T.), who was blinded to the experimental groups, were made by manually outlining the margins of the infarct areas and both hemispheres in each section. The area was calculated in five coronal sections, with the first section 3 mm posterior to the frontal tip. The area of infarction in each section was expressed as the percentage of the infarct area to the area of the contralateral hemisphere.[28]

VII. Measurement of fasudil concentration in CSF and blood

At the time of sacrifice, 0.2–0.4 ml of CSF was obtained from the cisterna magna of the animals and 5 ml of blood was taken for the measurement of fasudil concentrations. Fasudil levels in the CSF and blood samples were measured by high performance liquid chromatography (Beckman System Gold®; Beckman Instruments Inc., Fullerton, Calif., U.S.A.). Each sample (50 μl) was injected onto a Alltech Spherisorb ODS-25 micron column (25 cm × 4.6 mm) (Alltech Associates, Inc., Deerfield, Ill., U.S.A.). The column was run using isocratic eluent conditions (30% acetonitrile in H_2O, 0.05% trichloroacetic acid) and a flow rate of 1 ml/min. Fasudil eluted at 5.2 minutes as detected by its ultraviolet absorbance at λ = 320 nm using a Beckman 166 UV detector. Fasudil level was measured by comparing the peak area of fasudil samples to a standard fasudil curve.

VIII. Statistics

All data in this study are expressed as mean ± SD. A paired Student's t-test was used to compare the difference between the values obtained before and after treatment. A probability value of <0.05 was considered to indicate a significant difference.

Results

I. In vitro drug-release kinetics

The loading efficiency of fasudil was greater than 95% at a phospholipid:fasudil ratio of 1:0.4 (w/w), and liposomes routinely contained fasudil at a concentration of 140–160 μg fasudil/μmol phospholipid (0.43–0.49 μmol fasudil/μmol phospholipid). The release of fasudil from liposomes in CSF was biphasic. In the first 6 hours at 37°C, approximately 30% of the fasudil was released from liposomes (n = 3), and over 48 hours, 45% of the fasudil was released (Fig. 1).

II. Acute toxicity of fasudil following intrathecal administration

All rats that received free fasudil intrathecally showed considerable adverse effects. The rats receiving the two highest doses (0.25 or 0.10 mg free fasudil) both died. The rat that received 0.05 mg free fasudil showed abnormal posturing, but was still alive at 24 hours after intrathecal injection. The rat which received 0.025 mg free fasudil had a mild seizure, but quickly recovered. The rat which received the lowest dose (0.01 mg) was somewhat restlessness, but there were no other obvious adverse effects.

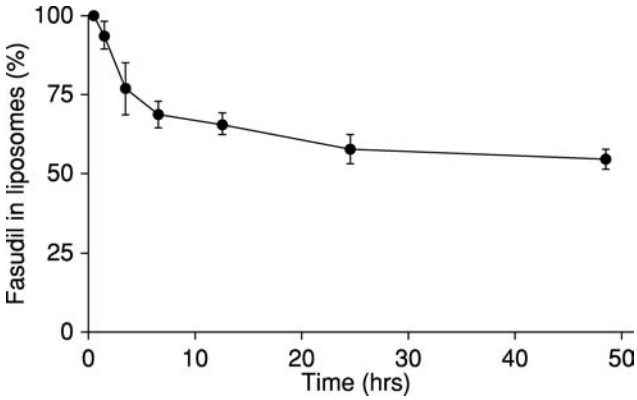

Fig. 1 Graph showing the cumulative percentage of fasudil released from liposomes into control cerebrospinal fluid in vitro at pH 7.4 and 37°C as a function of time (n = 3). Vertical bars represent SD. The half life of fasudil release in the α phase was 2.2 hours and in the β phase was 172 hours.

Table 1 Physiological variables in rats subjected to middle cerebral artery occlusion

	Control group (n = 10)	Treated group (n = 20)
Before occlusion		
Rectal temperature (°C)	37.4 ± 0.1	37.2 ± 0.2
pH	7.4 ± 0.03	7.4 ± 0.06
PO_2 (mmHg)	106 ± 8.9	118 ± 6.9
PCO_2 (mmHg)	38.1 ± 4.5	37.9 ± 4.1
MABP (mmHg)	93.1 ± 7.8	96.2 ± 8.6
After drug application		
Rectal temperature (°C)	37.2 ± 0.1	37.2 ± 0.04
pH	7.4 ± 0.01	7.4 ± 0.04
PO_2 (mmHg)	113 ± 10.6	111 ± 8.9
PCO_2 (mmHg)	38 ± 3.9	38.2 ± 4.6
MABP (mmHg)	95.6 ± 8.2	98.8 ± 9.2

Values are mean ± SD. MABP: mean arterial blood pressure.

III. Neurological deficits

In both control and treated groups, mean arterial blood pressure remained stable throughout the procedures and all other values were within normal ranges (Table 1). The control group, which received the drug-free liposomes, showed no adverse effect throughout the experiment. There was no significant difference between the neurological scores at 4 hours for all the animals within their respective groups (Table 2). At 24 and 72 hours after ischemic insult, animals treated with liposomal fasudil showed a better functional outcome with a significant improvement in neurological score when compared to those in the control group ($p < 0.001$).

IV. Evaluation of infarct damage

Application of an endovascular suture into the MCA successfully induced a well-defined region of ischemia as shown by the TTC-stained brain sections (Fig. 2). Lesions were noted in the dorsolateral frontoparietal cortex, the caudoputamen, and the globus pallidus. However, the region that should be supplied by the ACA was spared. In the current study, liposomal fasudil, at the non-toxic dose of 0.25 mg/rat, significantly reduced the infarct area when compared to that of the control group (24 hours: 29.0 ± 4.4%, 72 hours: 28.1 ± 3.9% vs. 49.6 ± 4.6%, respectively, $p < 0.001$). When the percentage of infarct area was calculated in each slice, the largest infarct area was found in slice 1, located 3 mm from the frontal pole.

V. Concentration of liposome-entrapped fasudil in CSF and blood

The concentration of liposome-entrapped fasudil in the CSF 24 hours after ischemic insult was 45.42 ± 31.48 μg/ml (n = 10). At 72 hours, technical difficulties prevented collection of adequate CSF.

Table 2 Neuroprotective effect of liposome-entrapped fasudil against neurological impairment

Neurological score*	Control group		Treated group		
	4 hrs (n = 10)	24 hrs (n = 10)	4 hrs (n = 20)	24 hrs (n = 10)	72 hrs (n = 10)
0	0	0	0	0	0
1	0	0	0	3	4
2	1	2	4	6	5
3	9	8	16	1	1
Average**	2.9	2.8	2.8	$1.8^{\#,\#\#}$	$1.7^{\#,\#\#}$

*Neurological scores are as follows: score 0, no observable deficit; score 1, forelimb flexion; score 2, forelimb flexion and decreased resistance to lateral push; score 3, forelimb flexion, decreased resistance to lateral push, and unilateral circling in three successive trials. **Total neurological score at 4, 24, and 72 hours after ischemic insult. $^{\#}p < 0.001$ vs. 24 hours control group, $^{\#\#}p < 0.001$ vs. 4 hours treated group. There was no significant difference in the treated groups at 24 and 72 hours.

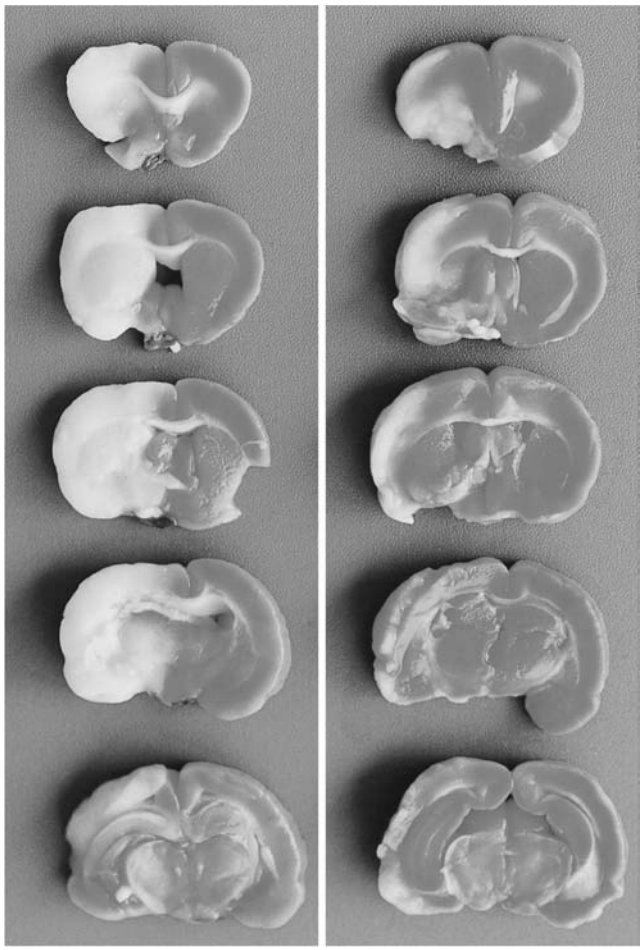

Fig. 2 Photograph of representative slices in the control (*left column*) and treated rats (*right column*) 24 hours following focal cerebral ischemia. The infarct area appears as unstained following staining with 2,3,5-triphenyltetrazolium chloride. Note the significant reduction of infarct area in each slice from the treated rat brain.

Fasudil was not detectable in the blood samples.

Discussion

Liposomes are composed of naturally-occurring, biodegradable lipids organized into bilayer lipid membranes enclosing an aqueous core. Liposomes of the composition (HSPC:CHOL) used in this study are non-toxic[14,25] and are widely used in anticancer agents.[2,11] Liposomes can be safely injected intracerebrally into the mouse[1] and rat.[12] In addition, the intra-CSF administration of liposome-entrapped drugs has been used in chemotherapy for brain tumors in rats,[19] monkeys,[18] and humans.[17] Therefore, we chose liposomes composed of HSPC:CHOL at 2:1 molar ratio. Our studies have confirmed that liposomal fasudil produced no obvious neurological or blood pressure changes following intrathecal administration in rats, at doses 10-fold higher than those that began to produce serious adverse effects for the free drug.

Effective treatment of cerebral ischemia requires that concentrations of the drug in the CSF be maintained within the therapeutic range. Under such circumstances, the advantages of using liposome-entrapped fasudil by intrathecal application are three-fold. First, liposomes cannot cross the blood-brain barrier. This keeps the drug confined primarily to the central nervous system, preventing adverse systemic effects. Second, encapsulation of fasudil in liposomes changes the pharmacokinetics of the free drug and provides sustained release of the drug with a half life in the α phase of 2.2 hours and a terminal half life of 172 hours. By comparison, free fasudil is cleared quickly from the blood with a half life of less than 15 minutes.[24] Thus, even if the free drug were administered by the intrathecal route, redistribution of the drug would quickly reduce its concentration below the therapeutic range. Third, although the morbidity associated with long-term intrathecal drug administration through indwelling catheters has precluded the practical application of these procedures in the clinical setting, the use of liposome-entrapped fasudil can avoid the problems related to continuous infusion. A sustained-release preparation of fasudil, small enough to be applied in the subarachnoid space at the time of lumbar puncture, could continuously deliver drug into the subarachnoid space without the risk of infection associated with externalized catheters or intravenous routes and, therefore, might be used prophylactically.

Many factors, including the liposome composition and size, the physicochemical properties of the drug, and the method of drug loading into liposomes will affect the drug release rate.[4] In our in vivo studies, injection of 0.25 mg liposomal fasudil resulted in a CSF concentration of liposomal fasudil of 45.4 $\pm$ 31.5 μg/ml after 24 hours in rats. The initial concentration in CSF was 238 μg/ml, based on the assumption that the CSF volume per body weight for rats is similar to that for humans, i.e., 3 ml/kg or 0.9–1.2 ml CSF in 300–400 g rats.[15,27] Therefore, approximately 80% of the fasudil was released from rat CSF in vivo in 24 hours. Over a similar time period, approximately 40% was released in vitro in control CSF. Although the clearance and elimination of liposomes in CSF are poorly understood, protein in the CSF might be responsible for accelerating the apparent drug release from liposomes in vivo. Alternative-

ly, the amount of liposomal drug in CSF may have been underestimated due to non-specific absorption of the liposomes, e.g. to the walls of the ventricles, which would result in an over-estimate of the release rate. Regardless of the cause of the discrepancy between in vitro and in vivo release rates, we have demonstrated that liposome-entrapped fasudil in the CSF acted as a depot preparation to achieve sustained release of drug at therapeutic concentrations without serious adverse effects. Depending on the leakage figure chosen, between 0.25 and 0.66 mg/kg/day of released fasudil can significantly ameliorate cerebral ischemia in a rat model. This dose is only 13% to 55% of the corresponding intravenous dosage range in humans of 1.2 to 1.8 mg/kg/day.

The mechanism of action of fasudil hydrochloride as an antivasospastic agent is thought to be inhibition of protein kinases A, G, and C and myosin light chain kinase, the final common pathway of smooth muscle contraction.[6] Fasudil functions as an intracellular calcium antagonist that has the potential to delay ischemia-induced intracellular calcium accumulation.[6] This direct mechanism to prevent excess intracellular calcium accumulation may be important in the neuroprotective effect of fasudil hydrochloride against ischemia.[5] Intravenous infusion of fasudil (1 mg/kg) for focal cerebral ischemia in gerbils is effective in reducing neurological deficits and loss of tissue potassium.[20] Fasudil may mitigate ischemic damage in the penumbral zone, possibly by ameliorating collateral blood flow and preventing calcium-related cell damage. Moreover, intravenous administration of fasudil in a microembolism model of rats prevents the accumulation of neutrophils, resulting in decrease of the infarct area and improvement of neurological functions.[23] In the present study, intrathecal application of liposomal fasudil directly into the CSF allowed diffuse distribution of the drug through the CSF pathways. If this is the case, liposomal fasudil small enough to be applied in the CSF may achieve a vasodilating effect and may be effective to prevent cerebral ischemia.

The current study showed that a single intrathecal injection of the liposome-entrapped fasudil is effective at preventing cerebral ischemia for at least 24 hours post-administration. Conventional pharmacological approaches to the treatment of acute cerebral ischemia have been plagued in part by the inability to either attain or maintain an adequate therapeutic drug concentration in the CSF, so the intrathecal use of a liposomal drug with sustained release characteristics may be beneficial in patients with acute ischemia. However, the present study cannot clarify the therapeutic window, because there was no significant difference in the treated groups at 24 and 72 hours after ischemic insult, possibly as 80% of fasudil was released from the liposomes within the first 24 hours. Given the drug release profile of the current formulation of liposomal fasudil in the CSF, it is likely that neuroprotection may be extended to several days. The drug release rate can be further manipulated by changing the liposome composition if a faster or slower release rate are deemed desirable. Further work will be required to identify the site of action, the therapeutic window, and the therapeutic index of the drug. Although we have confirmed that liposome-entrapped fasudil can be safely applied in the CSF in rats, the circulation and kinetics of the drug in the subarachnoid space still remain to be understood. Further investigations must be employed to clarify the pharmacokinetics of the drug in the CSF.

Acknowledgments

This work is supported in part by a grant from Yokohama Foundation for Advancement of Medical Science in Japan to Dr. Y. Takanashi, MRC grant MT-9127 to Dr. T. Allen.

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Commentary

The authors described the protective effect of fasudil against cerebral ischemia induced by middle cerebral artery occlusion in rats. Fasudil was entrapped in liposomes and intrathecally administered. Animals treated with a single intrathecal dose had a significantly smaller infarct area at 24 and 72 hours after ischemia than control animals. Free fasudil injected intrathecally induced significant toxicity, but liposome-entrapped fasudil showed no obvious toxicity at a 10-fold higher dose. Concentration of fasudil in CSF seemed to be maintained for a relatively long time. In this study, the authors observed the effect up to 72 hours after ischemia. More prolonged observation is needed to know the true protective effect of fasudil on ischemia. Distribution of fasudil in the CSF and brain should also be examined.

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The goal of a medical therapy to limit the devastating effects of acute ischemic stroke has not yet been realized in clinical practice. This article, however, offers significant hope that eventually we may be able to find an agent that can be given after the event that will limit the extent of stroke and the ensuing neurological deficit. In this paper the authors have used a novel method of delivery of a protein kinase inhibitor fasudil. Their strategy is to incorporate fasudil into liposomes as a drug delivery system, and then inject the liposomes into the cisterna magna to provide slow release of fasudil into the CSF. Not only have they demonstrated that this delivery system is safe and effective in providing the delivery of a drug over a period of time, in their experimental model of stroke in the rat they have demonstrated significant improvement in neurological outcome in size of stroke. This is an intriguing concept and one that should be developed future with the hope that it may provide a real benefit for stroke victims.

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The authors have used liposome-entrapped fasudil hydrochloride in a rat model of ischemia in attempts to limit infarct size. They determined that infarct size could be reduced by greater than 25 percent at both 24 and 72 hours. Concentrations of liposome-entrapped fasudil were measured and showed to be 20 percent of the injected dose at 24 hours. Fasudil is a known neuro-protective agent but its intrathecal usage has been the subject of some controversy because of previously noted toxicity. However, the authors have clearly shown that using liposomes, a novel delivery method of fasudil could be achieved. The infarct size reduction is very evident in the figures provided. The utility of liposomes here have clearly supported the proof of principle the authors were seeking. I was pleased to read this report about liposome delivery of a neuroprotective agent. This expands the repertoire of conditions that can be treated through drug delivery taking advantage of liposome technology. I congratulate the authors on their paper.

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This paper presents neuroprotection by intrathecal application of liposome-entrapped fasudil in a rat model of ischemia. Liposomes are artificial lipid bilayer vesicles and considered to be a useful drug delivery system. Indeed, liposomes have many advantages as a delivery system — they can be used to transfer drugs, enzymes and other biologically important molecules such as DNA and RNA into cells, they can protect their contents from interaction with plasma and cerebrospinal fluid (CSF) components, and they can alter the pharmacokinetics and biologic distribution of free compounds. Therefore, therapeutic strategy using liposomes may become attractive for a lot of diseases such as cerebral ischemia, neoplasms or degenerative diseases. On the other hand, fasudil is one of the most promising drugs to be able to prevent ischemic-induced neuronal damage. This paper demonstrated that a single intrathecal injection of liposomal fasudil could maintain a therapeutic drug concentration in the CSF over a period of time and significantly decreased infarct size in a rat model of acute ischemia. The experimental results are very good, but unfortunately analyses of pharmacokinetics on the liposomes in intrathecal space and drug-releasing mechanisms are not enough. These are the most important factors to determine whether the liposomal system is useful in patients with acute ischemia. Also, liposomes have various types, such as small unilamellar vesicles (SUV), multilamellar vesicles (MLV) or large unilamellar vesicles (LUV). Each liposome has different characteristics even if their components are same. Therefore, the authors need to perform further examination on the pharmacokinetics and selection of liposomal types. If these points are confirmed, this paper will become more persuasive. I hope further investigations will lead to clinical trials.

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