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# The Efficacy of Retrograde Infusion with LY231617 in a Rat Middle Cerebral Artery Occlusion Model

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**ABSTRACT: Background and Purpose:** We examined the efficacy of the antioxidant LY231617 administered five hours following middle cerebral artery (MCA) occlusion in rats. **Methods:** The treatment was contrived for a two hour interval. Group A (n=16) was left untreated. Group B (n=16) received an intravenous infusion of LY231617. Group C (n=6) received saline (86 µl/min) by retrograde infusion of the cerebral vein (RICV). Group D (n=22) was administered LY231617 (10mg/kg/2hr) in saline (86µl/min) by RICV. Local cerebral blood flow with [<sup>14</sup>C]-iodoantipyrine and blood-brain transfer constant with <sup>14</sup>C-α-amino-isobutyric acid were examined. Early ischemic damage was histologically examined with cresyl violet and Luxol fast blue and with triphenyl-tetrazolium chloride. **Results:** The results revealed a marked increase in local cerebral blood flow (over 600%, p < 0.01) after RICV with LY231617, with a significant improvement of BBB permeability in rats from group D. Ischemic brain damage measured with Luxol fast blue and triphenyl-tetrazolium chloride methods showed a significant improvement (50-91%) of ischemic damage in group D, as compared to groups B and C. **Conclusion:** Retrograde infusion of the cerebral vein with LY231617 resulted in a significant amelioration at seven hours post MCA occlusion.

**RÉSUMÉ: Efficacité d'une infusion rétrograde de LY231617 chez le rat comme modèle expérimental d'occlusion de l'artère cérébrale moyenne. Introduction:** Nous avons évalué l'efficacité du LY231617, un antioxidant, injecté 5 heures après l'occlusion de l'artère cérébrale moyenne (ACM) chez le rat. **Méthodes:** La durée du traitement était de deux heures. Le groupe A (n=16) n'était pas traité. Le groupe B (n=16) a reçu une infusion intraveineuse de LY231617. Le groupe C (n=6) a reçu du salin (86 µl/min) par infusion rétrograde dans la veine cérébrale (IRVC). Le groupe D (n=22) a reçu le LY231617 (10 mg/kg/2hrs) dans du salin (86 µl/min) par IRVC. Nous avons évalué le flot sanguin cérébral local (FSCL) au moyen de la [<sup>14</sup>C]-iodoantipyrine et la constante de transfert hémato-encéphalique au moyen de l'acide [<sup>14</sup>C]-α-amino-isobutyrique. Le dommage ischémique précoce a été évalué par coloration histologique au violet de crésyl et au bleu de Luxol (CL) et au chlorure de triphényl-tétrazolium (CTT). **Résultats:** Nous avons constaté une augmentation marquée du FSCL (plus de 600%, p<0.01) après l'IRVC au LY231617, accompagnée d'une amélioration significative de la perméabilité de la barrière hémato-encéphalique chez les rats du groupe D. L'évaluation des dommages ischémiques cérébraux par CL et CTT a montré une amélioration significative (50-91%) des dommages ischémiques dans le groupe D par rapport aux groupes B et C. **Conclusion:** Le traitement par IRVC de LY231617 a entraîné une amélioration significative 7 heures après l'occlusion de l'ACM.

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Despite numerous experimental studies for the treatment of acute focal cerebral ischemia, efficacy was limited by the timing of administration of cytoprotective agents either before or less than two hours after occlusion.<sup>1-3</sup> Clinical trials for the initiation of treatment of early acute stroke revealed that commencement began 1.5 to 5 hours following clinical onset of stroke by an emergency trauma team.<sup>4</sup> This study further examines the retrograde infusion of a cerebral vein (RICV) as a means of delivering cytoprotective agents with capillary vasodilator more efficiently and selectively to ischemic tissue.<sup>5,6,47</sup> Our previous study indicated that starting treatment with the Ca<sup>2+</sup> antagonist verapamil using RICV three hours after occlusion of the middle cerebral artery (MCAO), resulted in significant improvement in

local cerebral blood flow (LCBF) with reduction of ischemic brain damage.<sup>7</sup> Systemic administration of verapamil in the same model failed to provide any beneficial effect; in fact, treatment was associated with increased blood-brain barrier permeability (BBB) and edema.<sup>8</sup> In further studies, treatment

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using RICV with a combination of drugs, such as verapamil, dexamethasone and mannitol resulted in a significant therapeutic effect with treatment initiated five hours following MCA occlusion in rats.<sup>8</sup>

An excess of free radical production has been well documented to have potentially damaging effects on ischemic brain tissue.<sup>9-19</sup> Recent reports of free radical scavengers or antioxidants indicated that these drugs had a significant beneficial effect on ischemic neuronal damage. Superoxide dismutase has been reported to protect brain cells against focal and global ischemia.<sup>20-22</sup> Dimethylthiourea and allopurinol were also reported to act as hydroxyl radical scavengers through xanthine oxidase inhibitors.<sup>23,24</sup> Furthermore, the lipid peroxidation inhibitor, tirilazad mesylate has been a beneficial effect on ischemic neuronal damage,<sup>25,26</sup> and brain edema.<sup>13,26</sup> In the studies described above, drugs were given intraperitoneally either before or immediately after vessel occlusion to obtain a beneficial effect. A new antioxidant LY231617 (2,6-bis(1,1-dimethylethyl)-4[[1-ethyl]amino]methyl] phenolhydrochloride) administered intravenously was reported to reduce ischemic damage in a 30 minute and three hour old focal ischemic rat model.<sup>12,48</sup> LY231617 inhibited iron-dependent lipid peroxidation, attenuated glutamate neurotoxicity and brought about the blockage in arachidonic cascade.<sup>12</sup>

In the present study, a delayed initiation of treatment within a therapeutic time window of five hours after MCAO by RICV with LY231617 is examined.<sup>4</sup> For evaluation of the efficacy of RICV with LY231617, LCBF, alterations in BBB permeability, as well as extent of ischemic cerebral damage using a direct and indirect methods were examined.

## MATERIALS AND METHODS

Sixty Sprague-Dawley rats weighing 350-430g were fasted overnight with water provided ad libitum before the experiment. All rats had a focal cerebral ischemia brought about by occlusion of the left middle cerebral artery with electrocoagulation of the lenticulostriate branches after left subtemporal craniectomy using the modified method of Tamura et al.<sup>27</sup> The rats were then assigned to four groups labelled A through D. The untreated control group A (A=16) was studied for LCBF and BBB permeability, and early ischemic brain damage using direct histopathological methods, cresyl violet and Luxol fast blue (CL). The rats in group B (B=16) were treated for two hours intravenously with LY231617, five hours after the left MCAO. LCBF was examined and ischemic brain damage was analyzed directly using the CL staining method and indirectly using 2,3,5-triphenyl-tetrazolium chloride (TTC) staining. The rats in group C (C=6) were treated by RICV with saline (86µl/min) at the body temperature of 37°C which was started five hours after MCAO for a period of two hours. The rats were examined for ischemic brain damage by indirect measurement of TTC staining. Treatment of group D (D=22) with LY231617 in the saline of the body temperature of 37°C as the group C using retrograde infusion of the cerebral vein (RICV) (10mg/kg/2hr and 86µl/min) was started five hours after MCAO for a period of two hours. The rats in group D were examined for LCBF and BBB permeability. Ischemic brain damage was evaluated directly by histopathological examination by the CL staining method and indirectly by the semiautomated measurement method of TTC. <sup>11</sup>C-iodoantipyrine

(IAP) (specific activity; 50mCi/mmol) was used for measurement of LCBF and <sup>14</sup>C- $\alpha$ -aminoisobutyric acid (AIB) (specific activity; 58mCi/mmol) for measurement of the BBB permeability and 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma Chemical Co., St. Louis, MO., USA) was used for the determination of mitochondrial dehydrogenase activity. Antioxidant LY231617 was provided by the Lilly Research Laboratories, Eli Lilly Co., Indianapolis, Indiana.

Details of the surgical preparation and monitoring of physiological parameters have been previously published.<sup>5-8</sup> Briefly, anesthesia was induced by inhalation of 2.5-3% halothane for two to three minutes and maintained by spontaneous respiration with the delivery of 1.0-1.5% halothane in mixture with room air through a mask. Polyethylene catheters were placed in the femoral artery for monitoring blood pressure, blood gases, hematocrit, blood glucose and radioisotope concentration, and in the femoral vein for administration of radioisotope and drugs.

A dental drill was used to make a small craniectomy in the left temporal bone in all rats. All lenticulostriate branches of the left MCA were electrocoagulated. The left MCA was then occluded proximal to the lenticulostriate branches by a Zen clip (Ohwa Tsusho Ltd., Tokyo, Japan) and electrocoagulated just distal to the Zen clip using a modified Tamura method.<sup>27</sup> After occluding the left middle cerebral artery (MCAO) an additional small craniectomy was performed using a dental drill on the left squamous bone just behind the postglenoid foramen in rats of groups C and D. The inferior cerebral vein was exposed, and a tapered tip of a polyethylene catheter (PE-10) was cannulated backwards into the inferior cerebral vein to establish unidirectional flow into the ischemic tissue without any perfusion flow into the distal part of the inferior cerebral vein. After completion of the surgical procedure, all rats were immobilized in loose-fitting plaster casts, and halothane was discontinued. Lidocaine hydrochloride was used for local anesthesia of the surgical wounds in all groups. Within one hour after cessation of the general anesthesia, all rats awakened completely.

LY231617 was diluted in normal saline and infused at a rate of 86 µl/min into the femoral vein of rats in group B. The rats in group C received body temperature (37°C) of saline (86 µl/min) through the inferior cerebral vein using an infusion pump (IVAC 710 syringe pump, IVAC Corp., San Diego, California). The rats in group D were given LY231617 in the body temperature (37°C) of saline (86 µl/min) through the inferior vein using an IVAC infusion pump. All rats except for the untreated control group were infused over a period of two hours following five hour focal ischemia. During the two hours of RICV, the infusion pressure of saline and LY231617 solution was monitored constantly and kept at 150 mmHg.<sup>6</sup> Physiological parameters, such as systemic blood pressure, blood gases, hematocrit, and blood glucose were measured regularly. The body temperature was maintained at 37 ± 0.5°C with a heat lamp.

## Measurement of LCBF

We measured the LCBF in ten rats from each of Groups A, B and D using [<sup>14</sup>C]-iodoantipyrine (<sup>14</sup>C-IAP) at the end of the treatment. 30 µCi <sup>14</sup>C-IAP in 1 ml of normal saline was injected into the femoral vein using a Sage 351 syringe pump (Orion Research Incorp., Boston, MA) for a period of one minute. Arterial blood samples (20 µl each) were collected at five-second intervals after the start of injection of <sup>14</sup>C-IAP. At the end of the

one minute infusion the animals were decapitated. The brains were removed and immediately frozen in liquid Histo Freeze (Fisher Scientific., Nepean, Ontario, Canada). The brains were sliced into 20  $\mu\text{m}$  sections in a cryostat ( $-22^\circ\text{C}$ ) and each section was mounted on a microscopic cover glass and rapidly dried on a hot plate for autoradiography. The mounted brain sections were exposed on Kodak SB-5 films (Rochester, New York) for one week with [ $^{14}\text{C}$ ] standards (American Radiolabelled Chemicals Inc., St. Louis, Missouri). The densitometric measurements were made with a digital analyzer (The Image Calculator, McGill University, Canada). The mean values of the [ $^{14}\text{C}$ ] tissue radioactivities were obtained from each autoradiogram by measuring the optical density of three regions of one locus in three consecutive brain slices as designated by the rat brain atlas.<sup>28</sup> The radioactivity of [ $^{14}\text{C}$ ] was measured using a 1219 Rackbeta liquid scintillation counter (Wallace Oy, Turku, Finland). LCBF was calculated using the operational equation described by Sakurada et al. 1978.<sup>36</sup> The anatomical identifications of each area was made from the atlas of Paxinos and Watson.<sup>28</sup>

### Quantitative Assessment of Early Ischemic Brain Damage

#### A. Direct Histopathological Method using Cresyl Violet and Luxol Fast Blue (CL)

Seven frozen coronal tissue sections were taken from the ten rats used for LCBF measurement of groups A, B and D. The coronal tissue was sliced at 20  $\mu\text{m}$  thickness adjacent to that used for the  $^{14}\text{C}$ -IAP autoradiograms at 1.28 mm intervals beginning at a level 3.2 mm anterior to the occipital pole. These sections were dried, then fixed in 10% formaldehyde solutions overnight and stained by a combined method of cresyl violet and Luxol fast blue.<sup>29</sup> The areas of unstained ischemic damage were manually outlined on the digitized images of seven equally spaced coronal sections using a digital image analysis system (The Image Calculator, McGill University, Montreal, Canada). Any indistinct border of ischemic cerebral damage was examined under the light microscope for neuronal ischemic damage as described by Osborne et al. 1987.<sup>29</sup> The total volumes of ischemic damage were determined by the integration of areas with distance between neighboring sections.

#### B. Indirect Method Using Semiautomated Analysis of TTC Infusion

In separate experiments, a total of 18 rats, six from each of groups B, C and D were subjected to further quantitative examination of early cerebral ischemic damage by a more objective method using the TTC staining technique. The staining action of tetrazolium salts was based on the presence of active dehydrogenases in mitochondria.<sup>31</sup> Tissue with a normal level of enzyme activity was stained deep red, whereas the ischemic damaged tissue was unstained owing to lack of the mitochondrial enzyme activity.<sup>30</sup> The intracardiac infusion method of TTC was established to be superior to the immersion method.<sup>31</sup> The 2% solution of TTC was prepared with 37°C phosphate buffered saline (pH 7.4) immediately before use. Seven hours after the MCAO, anesthesia was induced by inhalation of 2 - 3% halothane and maintained by spontaneous respiration. An 18-gauge needle was passed through the left ventricle to the proximal ascending aorta under thoracotomy. The right atrium was incised, the descending

aorta was clamped, and 300 ml of the TTC solution was immediately infused by gravity flow through the needle for 30 minutes. During infusion, the temperature was maintained at 37°C using a heat lamp. After infusion for 30 minutes, the brains were immediately removed and fixed in 10% formalin solution overnight. This intracardiac perfusion of TTC staining method has been found to be more sensitive and reliable technique for detection of the early ischemic damaged cerebral tissue rather than the intravenous TTC staining method.<sup>31,34</sup> After fixation, the TTC-stained brain was sliced coronally using a thickness of 2 mm. The coronal slices were taken at 3, 5, 7, 9 and 11 mm from the occipital pole. The volume of ischemic damage were taken from all five coronal sections of color slide and measured by a digital imaging analysis system (MCID System; Imaging Research Inc., Ontario, Canada) adopting a semiautomated method described by Swanson et al.<sup>18</sup> The low optimal densities [mean value minus three times of standard deviation (less than 99.7% probability)] in the cortical gray matter and basal ganglia, excluding the white matter in the non-ischemic hemisphere were determined for each section by visual inspection and by automated densitometric measurement. These optical densities were then used as the threshold values for normal gray matter in the non-ischemic hemisphere by the image analysis system. These thresholds were established for each section to control for any variation of staining intensity or thickness. The areas of optical density greater than the threshold values were automatically measured by the image analysis system in the ischemic cerebral hemispheres of each section (Figure 4A and B). The volumes were calculated by multiplying each sum by the distance between sections. The volumes of the ischemic damaged tissue in the ischemic hemisphere were expressed as a percentage of the volume of the structures in the non-ischemic hemispheres.

$$A_D(\%) = (AN - AI) / AN \times 100$$

$A_D$  = percent of volume of ischemic damaged tissue in the ischemic cerebral hemisphere

$AN$  = volume of the non-ischemic hemisphere

$AI$  = volume of above the threshold tissue in the ischemic cerebral hemisphere

The direct measurement of early ischemic damage by the CL method was often associated with an overestimation of ischemic damage due to brain edema and swelling in the seven hour old focal ischemia. This artifact could be reduced by indirect, semi-automated analysis with TTC which was based on measured volumes of non damaged tissue in the ischemic hemisphere.<sup>32</sup>

#### Measurement of Blood-Brain Transfer Constant (Ki)

We employed the quantitative autoradiographic method developed by Blasbert et al.<sup>33</sup> to study the cerebral microvascular permeability in six rats from each of groups A and D. Six hours and 29 minutes after MCAO (30 minutes before the termination of the experiments), 30  $\mu\text{Ci}$  of  $^{14}\text{C}$ -AIB in 1 ml of normal saline was injected into the femoral vein using the Sage 351 syringe pump for a period of one minute. 50  $\mu\text{l}$  arterial blood samples were drawn at 0.25, 0.5, 1, 2, 3, 5, 7.5, 10, 15, 20, 25 and 30 minutes after the start of injection of  $^{14}\text{C}$ -AIB and immediately centrifuged. 20  $\mu\text{l}$  of plasma was then pipetted from each sample into counting vials. The rats were decapitated seven hours after MCAO and the brains were removed and immediately frozen. The brain sections were made with the

similar autoradiographic method as for LCBF measurement. Autoradiograms were exposed for a period of three weeks to the SB-5 films with  $^{14}\text{C}$ -standards. The densitometrical analysis of autoradiograms was performed with the similar procedures as for LCBF measurement. The (Ki)s for each locus were calculated from the  $^{14}\text{C}$  tissue concentration obtained from the autoradiograms and the  $^{14}\text{C}$  arterial plasma concentration-time integral.<sup>7</sup>

### Statistical Analysis

All data were expressed as mean  $\pm$  standard deviation. The statistical analysis of all data was performed using one-way analysis of variance (ANOVA), followed by Tukey's intergroup comparison test. A p-value  $< 0.05$  was considered significant.

## RESULTS

### Physiological Data

In all rats, blood pressure, blood gases, body temperature, hematocrit and blood glucose concentration were stable and did not change significantly during the MCAO nor during the systemic infusion or RICV of LY231617 (10mg/kg/2hours).

### LOCAL CEREBRAL BLOOD FLOW

The LCBF of the three groups is summarized in Table 1 and

Figure 1. The LCBF of each cortical and subcortical structure in the ischemic cerebral hemisphere of rats in group D were significantly increased (sensorimotor cortex: over 358%  $p < 0.01$ , anterior parietal cortex: over 680%,  $p < 0.01$ , posterior parietal cortex: over 286%,  $p < 0.01$ , caudate nucleus: over 316%,  $p < 0.01$ ; Table 1) compared to corresponding areas in groups A and B (Figure 1). There was no significant difference of LCBF in the ischemic cerebral hemisphere in group B as compared to group A (Table 1).

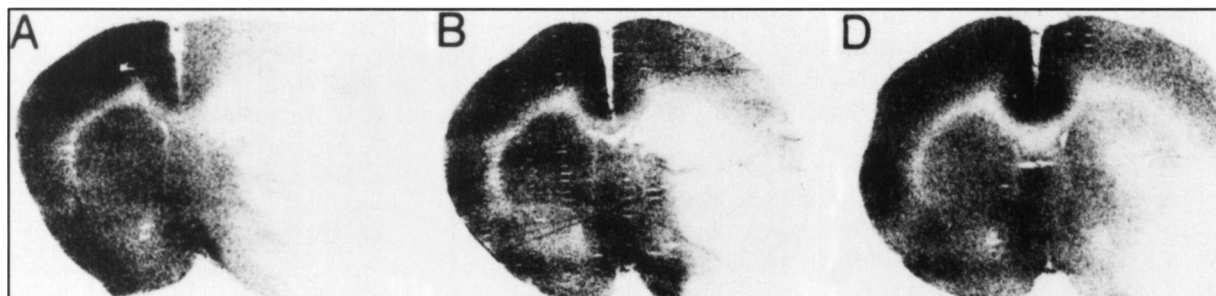
### Early Ischemic Brain Damage

#### A. Direct Measurement by the CL Method

In the quantitative volumetric measurement of early ischemic brain damage by the direct method using cresyl violet and Luxol fast blue, the rats in group D showed a significant reduction of over 31%,  $< 0.01$  (Table 2; Figure 1) in the total volume ( $\text{mm}^3$ ) of the ischemic hemisphere ( $166.8 \pm 6.7$  to  $102.4 \pm 9.3$ ,  $p < 0.01$ ), at sensorimotor level (over 34% reduction;  $p < 0.01$ ), at anterior parietal level (42% reduction;  $p < 0.01$ ), and at posterior parietal level (56% reduction;  $p < 0.01$ ) as compared with rats in groups A and B. Rats in group B showed no significant difference in total volume of ischemic damage as compared to group A (Table 2).

#### B. Indirect Measurement by TTC Method

In Figure 2A, B and C, the TTC staining patterns are shown



**Figure 1A,B,C:** Autoradiographic images of  $^{14}\text{C}$ -iodoantipyrine in a coronal section of sensorimotor cortex and caudate nucleus region. Retrograde infusion of the cerebral vein (RICV) with LY231617 Group (D) showed marked improvement of LCBF in the ischemic cortical and subcortical areas as compared to that of untreated (A) and LY231617 administered intravenously (B).

**Table 1:** Changes in LCBF Found After Starting Treatment Five Hours After MCAO for a Period of Two Hours.

Group	Group A (Untreated) (ml/100g/min) (n=10)		Group B (IV with LY231617) (ml/100g/min) (n=10)		Group D (RICV with LY231617) (ml/100g/min) (n=10)	
	Left	Right	Left	Right	Left	Right
Frontal Cortex	30 $\pm$ 7	131 $\pm$ 5	42 $\pm$ 7	115 $\pm$ 7	55 $\pm$ 7	115 $\pm$ 8
Sensorimotor Cortex	7 $\pm$ 2	124 $\pm$ 5	12 $\pm$ 3	110 $\pm$ 7	43 $\pm$ 7#	112 $\pm$ 9
Anterior Parietal Cortex	3 $\pm$ 1	118 $\pm$ 5	5 $\pm$ 3	107 $\pm$ 7	34 $\pm$ 7#	104 $\pm$ 10
Posterior Parietal Cortex	7 $\pm$ 3	119 $\pm$ 6	14 $\pm$ 5	108 $\pm$ 7	40 $\pm$ 7#	106 $\pm$ 10
Caudate						
Lateral	1 $\pm$ 0	122 $\pm$ 5	8 $\pm$ 3	108 $\pm$ 7	25 $\pm$ 10*	107 $\pm$ 9
Medial	11 $\pm$ 3	122 $\pm$ 6	18 $\pm$ 5	101 $\pm$ 7	70 $\pm$ 8#	102 $\pm$ 9
Postero-Lateral Portion of Caudo-Putamen	2 $\pm$ 1	109 $\pm$ 5	6 $\pm$ 2	100 $\pm$ 6	12 $\pm$ 3	93 $\pm$ 10

Values are stated as the mean  $\pm$  standard deviation

\*:  $p < 0.05$  significant difference from the Group A by ANOVA

#:  $p < 0.01$  significant difference from the Group B by ANOVA

**Table 2:** Direct Measurement of Ischemic Damage Using the CL Method in Five Hour Old Focal Ischemia Following Various Treatments.

Location	Group A (Untreated) (n=10)	Group B (IV with LY231617) (n=10)	Group D (RICV with LY231617) (n=10)
Sensorimotor Level (mm <sup>2</sup> )	36.9 ± 1.6	32.3 ± 2.5	21.2 ± 2.8#
Anterior Parietal Level (mm <sup>2</sup> )	18.8 ± 1.6	18.3 ± 1.7	10.9 ± 1.2#
Posterior Parietal Level (mm <sup>2</sup> )	14.1 ± 1.3	12.7 ± 1.9	5.6 ± 1.2#
Total Ischemic Damage Volume (mm <sup>2</sup> )	166.8 ± 6.7	148.5 ± 12.8	102.4 ± 9.3#

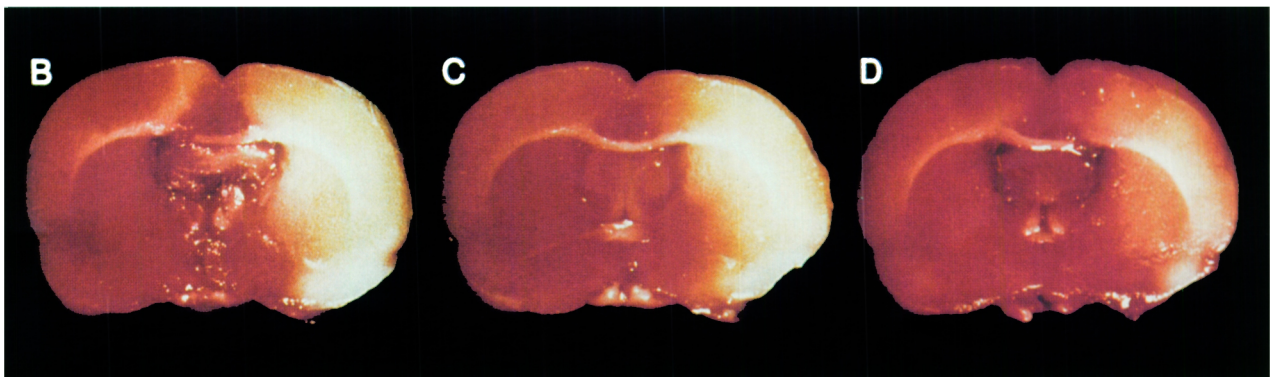
Values are mean ± standard deviation  
#: p > 0.01 significant difference between Groups A and B by ANOVA  
CL - cresyl violet and Luxol fast blue staining

at the sensorimotor level of each of groups B, C and D. At the sensorimotor level where the ischemic damage was at its maximum in the subcortical part of the striatum, a significant and marked reduction of ischemic damage tissue was observed in both cortical (over 76%, p < 0.005) and striatal (91%, p < 0.005) areas in group D, as compared to groups B and C. A total volume of ischemic damaged tissue was also significantly reduced to 52 - 57%, (p < 0.005) in group D as compared to group B (Table 3). This indirect, semiautomated analysis of ischemic damaged tissue eliminates the potential error and bias inherent in the manually delineating method of direct histopathological measurement using CL (Figure 4A and B).

Comparison of direct and indirect methods indicated that the measurement of a total volume of ischemic damaged tissue by the direct histological analysis of CL was over estimated by over 25% due to artifacts from the brain edema.

#### Blood-Brain Transfer Constant (Ki)

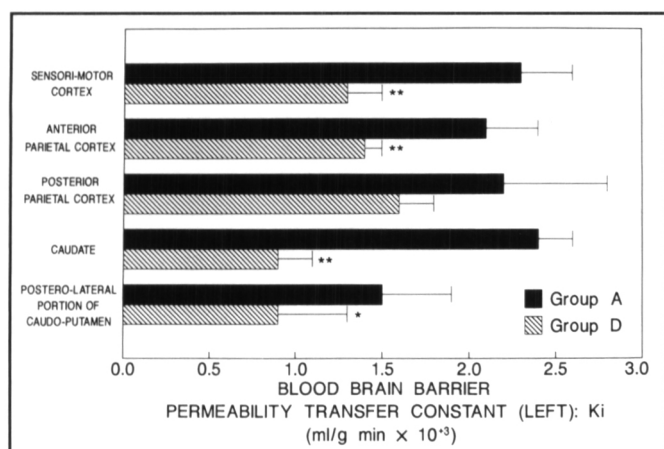
Ki values of BBB permeability changes were summarized in Table 4 and Figure 3. In comparison of Ki values between



**Figure 2A,B,C:** Coronal section of the sensorimotor cortex and caudate nucleus region, following intracardiac infusion of TTC. Intravenous infusion of LY231617: Group B showed 44.3% of cerebral tissue damaged (unstained with TTC) in the cortical area and 30.8% of tissue damaged in the striatum area of the ischemic hemisphere as compared to the non-ischemic hemisphere (B). Retrograde infusion of the cerebral vein (RICV) with saline: Group C showed 40.9% of cerebral tissue damaged in the cortical area and 31.4% of tissue damaged in the striatum area of the ischemic hemisphere as compared to the non-ischemic hemisphere (C). Retrograde infusion of the cerebral vein (RICV) with LY231617: group D showed only 11.7% of cerebral tissue damaged in the cortical area and 2.9% of tissue damaged in the striatum area of the ischemic cerebral hemisphere as compared to the non-ischemic hemisphere (D). There is a significant amelioration of cerebral tissue damage (52-57%, p < 0.005; p < 0.005) in Group D rats as compared to Groups B and C rats.

**Table 3:** Indirect TTC Analysis of Ischemic Damaged Tissue in Five Hour Old Focal Ischemia Following Various Treatments.

Damaged Tissue Volume (%)	Group B (IV with LY231617) (n=6)	Group C (RICV with Saline) (n=6)	Group D (RICV with LY231617) (n=6)
At the Sensorimotor Level			
Cortical Area	44.3 ± 4.3	40.9 ± 7.4	11.7 ± 4.6 (-76%, p < 0.005; against Group C) (-78%, p < 0.001; against Group B)
Striatum Area	30.8 ± 7.3	31.4 ± 6.0	2.9 ± 1.3 (-91%, p < 0.001, against Group C) (-91%, p < 0.005, against Group B)
Total Volume	22.5 ± 2.6	25.1 ± 2.6	10.9 ± 1.5 (-57%, p < 0.005, against Group C) (-52%, p < 0.005, against Group B)
Mean ± standard deviation (mm <sup>2</sup> ± SD)			

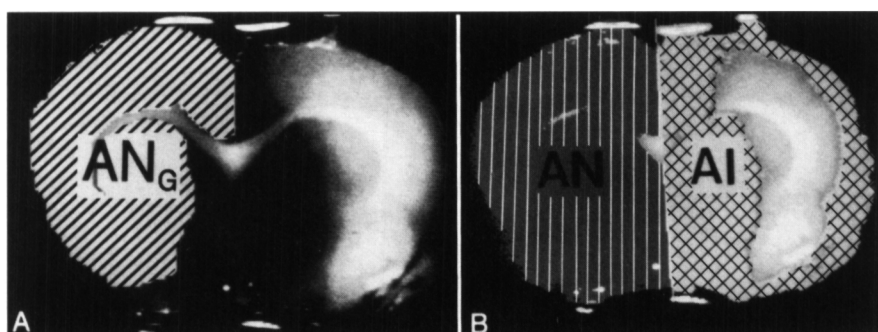


**Figure 3:** Histograms comparing blood-brain barrier permeability (Ki) values in ischemic cortical and subcortical areas. Retrograde infusion of the cerebral vein (RICV) with LY231617 (Group D) showed a significant reduction of changes in BBB permeability as compared to that of untreated animals (Group A). Columns and bars represent the mean ± standard deviation. \*:  $p < 0.05$ , \*\*:  $p < 0.01$  significant difference from control group by one way ANOVA.

ischemic hemispheres of groups A and D, there was a significant reduction of the Ki values in the cortices (sensorimotor cortex: 43%  $p < 0.01$  anterior parietal cortex: 33%  $p < 0.01$ ), and subcortical areas (caudate nucleus: 55%  $p < 0.01$ , posterolateral portion of caudoputamen: 40%  $p < 0.05$ ) in group D. Comparison of Ki values between ischemic hemisphere and non-ischemic hemisphere in Group A showed a significant increase of Ki values that was also observed in the ischemic cortices (sensorimotor cortex: 43%  $p < 0.05$ , anterior parietal cortex: 31%  $p < 0.01$ ) and subcortical area (caudate nucleus: 53%  $p < 0.01$ ) as compared to those of the non-ischemic hemisphere indicating that LY231617 has a protection and reparability of BBB function in the five hour old ischemic cerebral tissue.

**DISCUSSION**

LY231617 (2,6 - bis (1,1-dimethylethyl)-4-[[1-ethylamino] methyl] phenol hydrochloride) has the structure of butylated hydroxytoluene which has been known to have antioxidant activity. LY231617 and its related compounds were recently reported as having the following effects: inhibiting iron-depen-



**Figure 4A:** The diagram shows a coronal section at the sensorimotor and caudate nucleus level, seven hours after occlusion of the right middle cerebral artery, following the intracarotid infusion of TTC. The  $AN_G$  is the lowest optical density value (minus mean value  $\times 3$  standard deviation value) in the grey matter and basal ganglia, excluding the white matter in the non-ischemic hemisphere. This was determined for each section by the automated densitric measurement as the lowest threshold values ( $AN_G$  / / / / in Figure 4A) for recognition of normal grey and basal ganglia. These normal lower thresholds ( $AN_G$ ) were established for each section to control for varying section thickness and staining intensity. **B:** The area of non-ischemic damaged tissue with optical density greater than the threshold value was measured in both ischemic cerebral hemisphere ( $A_{N\#}$ ).  $A_D$  (%) =  $(AN - A_I) / AN \times 100$  in the percent volume of non-ischemic damaged tissue in the ischemic hemisphere.

**Table 4:** Comparison of Blood - Brain Transfer Rate Constant in the Control Group and RICV with LY231617.

Structure	Group A (Untreated) ( $\times 10^{-3}$ ml/g/ml) (n = 6)		Group D (RICV with LY231617) ( $\times 10^{-3}$ ml/g/ml) (n = 6)	
	Left	Right	Left	Right
Sensorimotor Cortex	2.3 ± 0.3@	1.6 ± 0.3	1.3 ± 0.2**	1.2 ± 0.3
Anterior Parietal Cortex	2.1 ± 0.3 @@	1.6 ± 0.3	1.4 ± 0.1@**	1.1 ± 0.2
Posterior Parietal Cortex	2.2 ± 0.6	1.6 ± 0.2	1.6 ± 0.2@**	1.2 ± 0.2
Caudate Lateral	2.4 ± 0.2@@	1.2 ± 0.3	0.9 ± 0.2**	0.8 ± 0.1
Caudate Medial	2.0 ± 0.2@@	1.3 ± 0.2	0.9 ± 0.2**	0.9 ± 0.2
Post. Lat. of C.P. Complex	1.5 ± 0.4	1.1 ± 0.3	0.9 ± 0.4*	0.7 ± 0.1

Values are expressed as the mean ± standard deviation

@@@:  $p < 0.05$ ; @@:  $p < 0.01$ , significant difference between non-ischemic and ischemic hemisphere in the Groups A and D by ANOVA; \*  $p < 0.05$ ; \*\*  $p < 0.01$ , significant difference in the ischemic hemisphere between Groups A and D by ANOVA

dent lipid peroxidation, attenuating glutamate neurotoxicity, blocking enzymes of the arachnoid cascade and ameliorated neuronal damage *in vitro* and *in vivo*.<sup>11,12</sup> This agent has an advantage of being water soluble and easily penetrates the BBB.<sup>11,12</sup> This agent also has a potent vasodilating effect and reparability of BBB function.

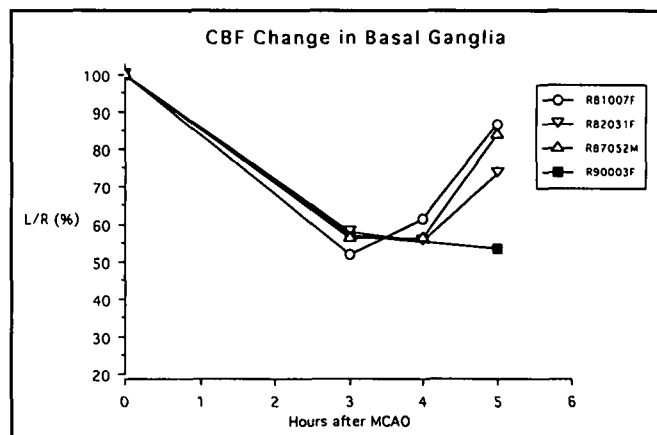
In the previous study, we observed a significant worsening of BBB permeability due to the vasodilating effect of RICV with verapamil in the seven hour old ischemic cortical areas. The values of BBB transfer constant (Ki) was significantly improved similar to the non-ischemic cortical areas with RICV with a combination of verapamil, mannitol and dexamethasone.<sup>8</sup> A comparison of beneficial effects between RICV with LY231617 alone and RICV with three combined drugs indicates that the antioxidant LY231617 alone resulted in the greater improvement of ischemic damaged tissue than the therapy involving the combination of all three drugs.<sup>8</sup> The body temperature (37°C) of saline was used in groups C and D with the same infusion rate, therefore, hypothermic effects were not expected a difference of therapeutic effect between group C and group D. We have studied the regional cerebral blood flow using oxygen-15 labelled H<sub>2</sub><sup>15</sup>O with high resolution positron emission tomography (PC-2048 system) during three to five hours after the occlusion of the left middle cerebral artery in Rhesus monkeys (Appendix 1).<sup>46</sup> This study indicates that CBF was further reduced during RICV with saline, but RICV with LY231617 was significantly increased in the same monkeys (unpublished)<sup>46</sup> due to potent vasodilating effect of LY231617 with marked vasodilating effect to the ischemic tissue in which reduced capillaries resist and produce "sinking effect" to the ischemic capillary to encourage the development of collateral flow from the non-occluded cerebral vessels and specific higher concentration (3 to 10 times) of LY231617 to the ischemic tissue.<sup>47</sup>

The accurate determination of the extent of ischemic damage in the ischemic cerebral hemisphere was essential for the assessment of therapeutic intervention and for the evaluation of therapeutic efficacy. Therefore, we used both a direct histopathological method using a CL staining technique and an indirect semiautomated method<sup>18</sup> using the TTC infusion technique to delineate accurately and objectively the areas and volumes of ischemic damage following various therapeutic procedures. The reliability of TTC staining as a marker of ischemic damage had been evaluated by comparing light and electron microscopic findings by several investigations<sup>30,31,34</sup> and it was found that TTC-staining could detect early ischemic damage and accurately delineate the extent of an ischemic area in over six hour old focal cerebral ischemia by intracardiac perfusion of TTC.<sup>31,34</sup> The indirect, semiautomated method with intracardiac perfusion of TTC provided an accurate and objective measurement of size and volume of early ischemic brain damage, particularly when the damaged tissue was compromised by edema and swelling.<sup>18,32</sup> This indirect semiautomated measurement of ischemic damaged volume, based on the measurement of residual non-damaged tissue in the ischemic hemisphere, eliminated the artifacts produced by brain edema and swelling in the ischemic damaged tissue.<sup>18,32</sup> The difference between the volume of the ischemic damage determined by both methods, indicated that the indirect method greatly reduced or eliminated error from brain swelling and added objectiveness to the analysis. Therefore, the present study indicated that treat-

ment using retrograde infusion of the cerebral vein (RICV) with LY231617 (Group D) provided a significant improvement of ischemic brain damage as compared with intravenous treatment (Group B) or retrograde infusion of the cerebral vein (RICV) with saline (Group C) in the seven hour old focal ischemic model.

The dose limiting toxicity of LY231617 is hypotension. Intravenous and retrograde infusion of the cerebral vein (RICV) infused at a rate of 10 mg/kg per two hours did not result in any significant reduction of the mean arterial blood pressure (MAPB). However, a dose of 20 mg/kg over a similar period resulted in a significant reduction of MABP with both intravenous and retrograde infusion of the cerebral vein (RICV). Therefore, a dose of 10 mg/kg/2hr was utilized for this study.

A peroxidative mechanism has been implicated as one of the prime detrimental factors in ischemic neuronal and vascular damages following cerebral ischemia.<sup>1,34,35</sup> Endothelial cells of brain microvessels were known to be primary targets for free radical injury,<sup>10,13,16,17,37</sup> because of the high concentration of xanthine oxidase, nitric oxide synthase.<sup>30,45</sup> Oxygen radical activities also cause severe vascular damage with disruption of BBB permeability and a resulting vasogenic edema.<sup>10,17,25,38,44</sup> Free radical scavengers or antioxidants were reported to prevent these cascades in endothelial cells to preserve capillary function.<sup>21,23,24,26,38,39</sup> In this study, the blood-brain transfer constant (Ki) was found to be significantly increased in the ischemic cortex (30 to 40%) and caudate nucleus (50 to 100%) as compared to the non-ischemic hemisphere in group A. The Ki values were markedly improved to non-significant degree between ischemic and non-ischemic hemispheres of both cortical and subcortical areas in group D. Therefore, there was significant improvement of BBB permeability disruption following retrograde infusion of the cerebral vein (RICV) with LY231617 treatment. There are limited reports of efficacy of the free radical scavengers on the LCBF after ischemia via systemic administration, the majority of which failed to reveal any significant improvement of LCBF except for these areas where collateral circulations existed.<sup>14,20,21,34,40</sup> Recent reports using free radical scavengers delivered intravenously for models of permanent MCAO indicated a significant efficacy on brain edema. These improvements were only obtained when these drugs were administered systemically shortly before or less than one hour after MCAO.<sup>14,26,39,41</sup>



Appendix 1<sup>46</sup>

A new antioxidant, LY231617, was recently reported to cause significant inhibition of iron-dependent lipid peroxidation, blocking glutamate neurotoxicity and reducing oxidative damage resulting from hydrogen peroxidation.<sup>12</sup> Inhibition of free radical activities by antioxidant LY231617 possibly plays an important part in the dramatic improvement of LCBF, BBB permeability, and ischemic brain damage in both ischemic cortical, as well as subcortical areas, by the retrograde infusion of the cerebral vein (RICV) delivery method. Thus the treatment using retrograde infusion of the cerebral vein (RICV) with LY231617 is a single agent capable of ameliorating and preventing various ischemic damaging mechanisms in the ischemic cortical and subcortical areas related to abundant collaterals.<sup>42</sup>

The retrograde infusion of the cerebral vein (RICV) is a minor neurosurgical intervention without any alteration of intracranial pressure<sup>43</sup> and minimum disturbance to the general cerebral venous circulation due to the abundance of collateral channels among the cerebral venous system. Using this minor neurosurgical procedure of retrograde infusion of the cerebral vein (RICV) with antioxidant, we can expect to achieve a significant improvement of focal ischemia even five hours after, in the focal ischemic model in rats.

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