tension is mild or moderate and no abnormal findings related to hypertension are observed, noncardiac surgery may not be postponed. However, patients with severe hypertension require careful blood pressure control throughout the perioperative period. In patients who cannot take drugs orally and who are going to undergo emergency surgery, intravenous infusion of antihypertensives is recommended.[72]

(3) Priority of Surgeries and Simultaneous Surgery
Abdominal aortic aneurysms (AAA) ≥6 cm in diameter should be treated surgically before noncardiac surgery even when the latter involves surgery of malignant disease, or should be treated concomitantly during noncardiac surgery in the case of abdominal surgery.[73,74]

Figure 3 shows the guidelines for priority of noncardiac surgery and TAA surgery. Priority depends on the pathological conditions of aortic aneurysms and noncardiac diseases. Further investigation of optimal management of aortic aneurysms in patients receiving noncardiac surgery is needed.

(4) Stent Grafting
Although stent grafting for aortic aneurysm is not a standard treatment procedure in Japan, and is only available in limited institutions, it may be used for the treatment of aortic aneurysms depending on the anatomical and structural characteristics of lesions. It has been found that stent grafting improves the short- and long-term prognosis of patients with relatively low risk of surgical complications who are likely to tolerate conventional artificial vascular graft implantation with thoracotomy or laparotomy, while it does not substantially improve the prognosis of high-risk patients.[75] Further clinical data and analyses are needed to determine the efficacy and safety of this technique. However, if outcome does not differ between endovascular stent grafting and surgical grafting, stent grafting is worth considering because it is useful, less invasive option to treat aortic aneurysms in patients undergoing elective noncardiac surgery to ensure a favorable clinical course during the perioperative period of noncardiac surgery.

(5) Rare Heart Diseases
i) Aortitis Syndrome (Takayasu Disease)
Surgical treatment is required in 13% of patients with aortitis syndrome. To ensure the safety of noncardiac surgery, patients should be carefully observed for hypertension due to renal artery stenosis and heart failure associated with aortic regurgitation. Patients with active inflammation as suggested by a high CRP level should be treated with corticosteroids to control inflammation and corticosteroid therapy should be reduced before noncardiac surgery when it is not urgently required.

ii) Marfan Syndrome
The outcome of cardiovascular surgery in patients with Marfan syndrome is excellent.[76,77] When appropriate cardiovascular evaluation does not reveal abnormal findings, patients with Marfan syndrome may usually undergo conventional noncardiac surgery.

(6) Management of Aortic Aneurysms in Patients With Other Heart Diseases
i) Ischemic Heart Disease
It has been reported that coronary lesions are observed in one-eights of patients with aortic dissection and one-thirds of patients with true aortic aneurysm. Ischemic heart disease is more common among patients with AAA.[72] Ischemic heart disease should be carefully managed during the perioperative period of patients undergoing aortic aneurysm surgery. IABP support is often not feasible in this patient population. Since hemodynamics during the perioperative period are often unstable due to hypertension and systemic arteriosclerosis, management of ischemic heart disease is more important during aortic surgery than other types of surgery.

Patients with aortic disease requiring elective, nonurgent surgery and symptomatic or severe coronary artery disease should undergo coronary surgery first, coronary surgery and aortic aneurysm surgery simultaneously, or coronary intervention first. DES, which require long-term treatment with potent antiplatelet drugs, are not feasible in patients planned to undergo aortic aneurysm surgery after coronary stenting. When simultaneous surgery is selected, hybrid treatment, namely, off-pump coronary bypass surgery and transcatheter coronary intervention should be considered. Patients with aneurysms in the descending aorta or thoracoabdominal aorta may simultaneously undergo aortic surgery and bypass surgery to the left anterior descending artery and/or the circumflex coronary artery.

ii) Valvular Heart Disease
When aortic surgery and valve surgery can be performed through the same incision, simultaneous surgery is feasible provided that cardiac function is normal. Although no consensus has been reached regarding the optimal surgical treatment of aortic lesions and valvular lesions which cannot be treated through the same incision, it is important to consider stent grafting as an option.

6. Peripheral Arterial Disease (Abdomen, Neck, and Lower Extremities)

Table 15 shows guidelines for evaluation and management of patients who have AAA, carotid artery stenosis, or arteriosclerosis obliterans (ASO) of the lower extremities and are to undergo noncardiac surgery under general anesthesia (cases of emergency surgery for noncardiac diseases are excluded). Since peripheral arterial disease may develop as a result of arteriosclerosis, patients diagnosed with a vascular lesion must be examined for other vascular lesions.

(1) Abdominal Aortic Aneurysms
When patients with AAA undergo noncardiac surgery, they should be carefully observed for rupture of aortic aneurysm, embolism due to mural thrombi, and blood coagulation disorder during the perioperative period. It is believed that noncardiac surgery before aneurysm surgery does not significantly increase the incidence of rupture. The indication for AAA surgery should be determined primarily without considering possible effects of noncardiac surgery, and the order of noncardiac surgery and AAA surgery should be based on the prognosis of AAA and noncardiac disease. Physicians should avoid performing gastrointestinal tract surgery and AAA surgery simultaneously since vascular grafts may be contaminated during surgery.

In cases of spindle-shaped aneurysms ≥6 cm in diameter, which are known to have a high risk of rupture, and of saccular aneurysms, symptomatic aneurysms, and infectious aneurysms regardless of the diameter, AAA surgery should be performed before noncardiac surgery or simultaneously with it. Although it has been reported that peripheral arterial embolism develops in 3 to 29% of patients with AAA,[78-80]

Table 15. Guidelines for Noncardiac Surgery in Patients With Peripheral Vascular Disease

Peripheral vascular disease	Treatment	Evaluation		
		Class I	Class II	Class III
AAA	Perform aortic surgery first (or simultaneously in some noncardiac diseases)	• Ruptured aneurysm • Symptomatic aneurysm • Spindle-shaped aneurysm ≥6cm of maximal diameter • Aneurysm with bleeding tendency • Infectious aneurysm	• Spindle-shaped aneurysm 5 to 6cm of maximal diameter • Saccular aneurysm • Rapidly expanding aneurysm • Aneurysm causing embolism	• Slowly expanding spindle-shaped aneurysm ≤5cm of maximal diameter
Extracranial stenosis of carotid artery	Perform carotid surgery first (or simultaneously in some noncardiac diseases)	• Symptomatic carotid stenosis ≥70%	• Symptomatic carotid stenosis 50 to 69%	• Symptomatic carotid stenosis ≤49% • Asymptomatic carotid stenosis
ASO of the lower extremities	Treat ASO of the lower extremities first (or simultaneously in some noncardiac diseases)		• Severe limb ischemia	• Intermittent claudication

AAA, abdominal aortic aneurysm; ASO, arteriosclerosis obliterans.

prediction of embolism is difficult. Patients with ecchymoses and prolonged hemostasis should be suspected to have consumption coagulopathy associated with aneurysms. The incidence of coagulopathy tends to be high in patients with large aneurysms, and it is difficult to control blood coagulation unless the aneurysm is surgically treated.

Patients with the following anatomical characteristics may undergo stent grafting for the treatment of AAA, and may thus undergo noncardiac surgery earlier than those undergoing conventional aneurysm surgery.

1) A 20 to 22 Fr catheter sheath may be inserted.
2) Infrarenal abdominal aorta (central landing zone) is 19 to 26mm in diameter and ≥15mm in length.
3) Infrarenal abdominal aorta with an angle of ≤60° relative to long axis of aneurysm.
4) Common iliac artery (distal landing zone) is 8 to 16mm in diameter and ≥10mm in length.
5) Absence of bilateral common iliac aneurysms.

(2) Carotid Artery Stenosis
The carotid artery should be checked in patients with a history of cerebral infarction and those suspected to be experiencing a transient ischemic attack (TIA). Patients with carotid artery stenosis are at risk for cerebral infarction during the perioperative period of noncardiac surgery. Since the risk of cerebral infarction is high in males, patients with a history of cerebral infarction rather than TIA, patients with cerebral hemisphere signs and symptoms rather than amaurosis, carotid surgery should be considered.[81,82] No benefit of carotid surgery has been observed in patients with mild stenosis with or without symptoms. Carotid endovascular treatment may be considered in patients with symptomatic severe carotid artery stenosis, those in whom the surgical approach to the carotid artery is difficult, those with a high risks associated with surgery, those with carotid artery stenosis after radiotherapy, and those with carotid artery restenosis after surgery. When noncardiac surgery is performed without treating carotid artery stenosis, patients should be managed carefully to prevent dehydration and hypotension and thus prevent cerebral infarction.

(3) Arteriosclerosis Obliterans of the Lower Extremity
Acute exacerbation of hemodynamics of the lower ex-

tremities is an important problem during the perioperative period of noncardiac surgery. Careful monitoring should be performed, particularly in patients with severe chronic leg ischemia whose blood pressure is of ≤50 to 70mmHg in the foot joint and ≤30 to 50mmHg in the toes.[83] When acute ASO of the lower extremity develops, amputation of the lower extremities may be required, or reperfusion injury followed by multi-organ failure may occur.
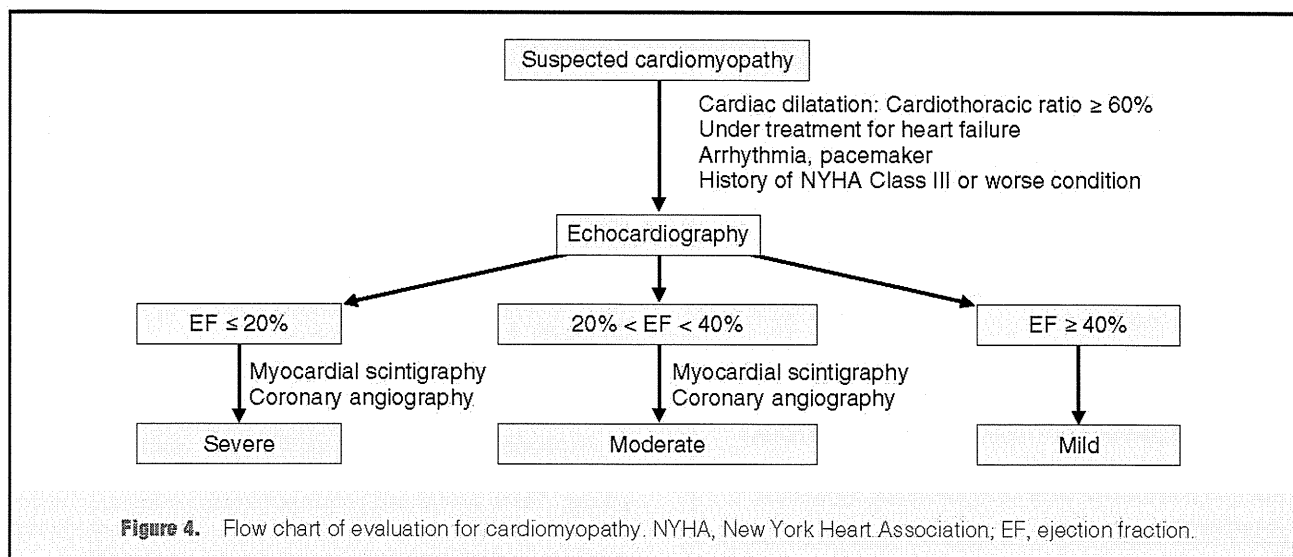
7. Pulmonary Artery Disease

Primary pulmonary artery disease that can coexist in paients undergoing noncardiac surgery are mainly idiopathic PH, familial PH, and chronic pulmonary thromboembolism, and these entities are discussed here.[84] When the following findings are noted in patients planned to undergo noncardiac surgery, they should be suspected to have PH and carefully evaluated for it.

Clinical findings:	Exertional dyspnea, leg edema, facial edema
Auscultation:	Pulmonary diastolic murmur and apical holosystolic murmur
Chest X-ray:	Left second arc protrusion and decrease in peripheral vessel shadow; left fourth arc protrusion
ECG:	Findings of right ventricular overload (S1Q3T3 and S1S2S3 patterns in typical cases)
Echocardiography:	Right ventricular enlargement, paradoxical motion of the interventricular septum

Pulmonary artery disease is strongly suspected when resting mean pulmonary arterial pressure in catheterization is ≥25mmHg, and hypocapnia with hypoxemia are observed on arterial blood gas analysis, while no significant pulmonary parenchyma diseases or airway disorders are observed on respiratory function testing. Prior to noncardiac surgery, physicians should consider that the natural history of moderate or severe PH is quite poor.

No systematic criteria are available to evaluate the risk of perioperative complications in noncardiac surgery in patients with PH. Since patients with PH tend to have hypoxemia and

Figure 4. Flow chart of evaluation for cardiomyopathy. NYHA, New York Heart Association; EF, ejection fraction.

right heart failure, careful monitoring (ECG, arterial line placement, and pulse oximetry) should be performed from the induction of anesthesia through the postoperative period. Although pulmonary arterial catheterization provides important information, it is difficult to place the catheter at an appropriate position, and lung injuries due to puncture and vessel injuries due to balloon dilatation may cause serious outcomes. Transesophageal echocardiography is very useful for monitoring the right ventricular function.

The effects of decreasing pulmonary vascular resistance during the perioperative period of noncardiac surgery with inhaled nitric oxide, dipyridamole, phosphodiesterase (PDE) III inhibitors, PGI₂, calcium blockers, and intravenous nitroglycerin have been reported. Endothelin-1 receptor antagonist are effective but not appropriate during the perioperative period, since only oral forms of them are available.

8. Idiopathic Cardiomyopathy

Cardiomyopathy is defined as "heart muscle disease associated with cardiac dysfunction", and is classified into dilated cardiomyopathy, hypertrophic cardiomyopathy, restrictive cardiomyopathy, arrhythmogenic right ventricular cardiomyopathy, and other unclassifiable cardiomyopathy. "Heart muscle disease with known etiology or clearly related to systemic disease" is defined as specific cardiomyopathy, and is not included into the above classification.[85,86] Although new classification have recently been proposed, this classification is still common in Japan and useful in the clinical setting. Figure 4 shows a flow chart of preoperative examinations for cardiomyopathy.

In management during the perioperative period of noncardiac surgery, arrhythmia and low cardiac output require special attention in patients with any type of cardiomyopathy. Extra vigilance is needed in patients with severe ventricular arrhythmia, which may cause sudden death. Patients often have been treated with antiarrhythmic drugs and may receive continuous intravenous lidocaine infusion during the perioperative period as needed, and many cases of arrhythmia are intractable. It is important to maintain normal sinus rhythm by adjusting electrolyte levels. If such treatment is impossible, heart rate should be controlled while in atrial fibrillation.

Low cardiac output in patients with dilated cardiomyopathy is treated by decreasing afterload with vasodilators and increasing cardiac contractile force with catecholamines and PDE III inhibitors, while such treatment is contraindicated in patients with hypertrophic cardiomyopathy and should be performed with care in patients with restrictive cardiomyopathy. Physicians should attempt to optimize intravascular volume to increase cardiac output regardless of the type of cardiomyopathy. However, since the range of the target intravascular volume is narrow, a pulmonary artery catheter should be placed to monitor hemodynamics carefully during the perioperative period, in which intravascular volume may change significantly, and diuretics should be administered whenever necessary.

Patients who had received warfarin to prevent embolism should be switched from warfarin to continuous infusion of heparin at least two days before surgery, and should discontinue heparin therapy about 3 hours before surgery. It is preferable that warfarin therapy be promptly resumed when the risk of postoperative bleeding has decreased, and that patients who cannot take drugs orally be administered heparin continuously. Adequate pain control is necessary, since postoperative pain increases afterload by increasing sympathetic activity.

9. Arrhythmias

In addition to myocardial infarction, arrhythmias and conduction disorders are quite common perioperative cardiac complications of noncardiac surgery. Arrhythmia may not occur as a single disorder. It is important to check for all possible heart diseases associated with arrhythmia when examining patients with perioperative arrhythmia.

(1) Perioperative Arrhythmia and Its Treatment
i) Preoperative Arrhythmia
When arrhythmia occurs in patients planned to undergo noncardiac surgery, physicians should check for the presence/absence of an underlying disease causing the arrhythmia and consider how to manage the patient should arrhythmia worsen during the perioperative period. The following types of organic heart disease may play roles in preoperative arrhythmia.

Table 16. The Causes of Arrhythmias That May Occur During the Perioperative Period

Myocardial ischemia

    Hypovolemia ← bleeding

    Hypoxemia ← pneumothorax, bleeding in pleural cavity, atelectasis, respiratory tract bleeding, etc.

    Coronary embolism (e.g., air embolism)

    Coronary spasm (hyperventilation, hypercalcemia)

Cardiac overload

    Atrial overload → atrial extrasystole, ventricular overload → ventricular extrasystole

    Overhydration (transurethral resection of prostate, burns, etc.)

    Pulmonary embolism (including air embolism, fat embolism) → right heart overload

    Mitral regurgitation, aortic regurgitation → left heart overload

Neurogenic arrhythmia (e.g., vagal impulse)

Electrolyte imbalance such as hypokalemia, hypocalcemia

Hypothermia

- Sick sinus syndrome, atrioventricular block (particularly Mobitz type II, Grade 3) → Coronary heart disease
- Ventricular extrasystole (multifocal, sequential) → Coronary heart disease, previous myocardial infarction
- Ventricular extrasystole → Cardiomyopathy, left ventricular hypertrophy/dilatation (valvular disease)
- Atrial fibrillation → Left ventricular diastolic dysfunction, valvular disease

There are reports suggesting that detailed monitoring and specific treatment are unnecessary in patients with preoperative ventricular extrasystole when myocardial infarction or other heart disease is absent.[87] However, since arrhythmia may worsen during the perioperative period in patients with ischemic heart disease, appropriate examination should be performed to exclude possible diseases and uncover undiagnosed diseases.

For patients who have been diagnosed with arrhythmia and are taking antiarrhythmic drugs, physicians should consult with anesthesiologists to determine whether antiarrhythmic drugs should be given intravenously or be suspended during the perioperative period. Many believe that β blockers used before surgery should be continued during the perioperative period.[19] In patients receiving anticoagulation therapy to control atrial fibrillation, physicians should consider the benefits and risks of bleeding with anticoagulation therapy in determining a strategy of treatment for the perioperative period.

ii) Arrhythmias That May Occur During Surgery

Table 16 lists conditions that may cause arrhythmias during the perioperative period. Although arrhythmias existing before surgery and underlying heart disease affect the type and incidence of arrhythmias during surgery, myocardial ischemia, overload on the heart, hypokalemia, and hypomagnesemia during surgery may induce arrhythmia. Anesthetics, surgical procedures, and bleeding control also affect the incidence of arrhythmia during surgery. Since cardiac arrest may occur at the time of reperfusion during surgical treatment of intestinal ischemia or lower extremity ischemia, appropriate measures such as exsanguination of venous blood may be required.

Intraoperative bradycardia may be improved for a short period of time with atropine sulfate and β agonists. However, when bradycardia is prolonged or severe, patients may need ventricular pacing using transvenous leads inserted from the internal jugular vein, transesophageal pacing, or external pacing using chest patch electrodes.

iii) Arrhythmias That May Occur After Surgery

The incidence of cardiac complications is highest during the first several days after surgery.[88] Arrhythmias that may occur after surgery include those immediately after recovery from anesthesia, fatal arrhythmias due to pulmonary embolism, which is prone to occur during the first several days after surgery, and atrial fibrillation, the incidence of which is high during the first week after surgery.

Atrial fibrillation is clinically significant, since thrombus may develop in the left atrium and cause arterial embolism. Transesophageal echocardiography is useful arterial to exclude possible arterial thrombus. Patients with atrial fibrillation may exhibit severe bradycardia requiring temporary pacing. Since severe and prolonged bradycardia may reflect the presence of latent conduction disorder, physicians should consider prompt implantation of permanent pacemakers.

(2) Perioperative Management of Patients Using Implantable Pacemakers and Implantable Cardioverter Defibrillators

In patients with implantable pacemakers and implantable cardioverter defibrillators (ICD), electromagnetic interference and infection are the most important complications of noncardiac surgery.

The use of electric knives may interfere with pacemakers, which will then not function properly. Unipolar devices are more susceptible to interference than bipolar devices. Physicians should be aware of the risk of electromagnetic interference when the surgical site is in close proximity to the pacemaker or leads. Physicians should also be familiar with the possible effects of use of electric knives at a surgical site distant from the pacemaker. Use of bipolar electric knives is in all cases the safest procedure, though such devices may make surgical procedures more complicated than unipolar devices. Pacing mode must be adjusted during surgery if surgical site is close near to the pacemaker and require frequent use of electric knives to stop bleeding. In patients who depend on a pacemaker to maintain heart rate, AOO, VOO, or DOO mode may be used during surgery. In patients in their own rhythm with the pacemaker in sense mode, the pacemaker is not used or is used with a low pacing rate during surgery.

In patients using an ICD, electromagnetic interference by electric knives may trigger the device, which may deliver a shock during surgery. In such patients, external patch electrodes should be placed on the chest wall to prepare for prompt electrocardioversion, and the ICD should be turned off during surgery. After surgery, the ICD should promptly be turned on. Continuous administration of antiarrhythmic drugs should be considered in patients susceptible to ventricular tachycardia. In any case, physicians and medical engineers with expertise in adjusting programs of implantable pacemakers and ICD should support the surgery.

In patients undergoing gastrointestinal surgery and patients with traumatic open wounds, bacteremia may develop. If leads exposed to venous blood become infected, the pacemaker may need to be removed. In patients with implantable pacemakers and ICD, antibiotic treatment should be initiated during surgery to minimize the occurrence of pacemaker infection.

References

1. Detsky AS, Abrams HB, McLaughlin JR, Drucker DJ, Sasson Z, Johnston N, et al. Predicting cardiac complications in patients undergoing non-cardiac surgery. *J Gen Intern Med* 1986; **1**: 211–219.
2. Ashton CM, Petersen NJ, Wray NP, Kiefe CI, Dunn JK, Wu L, et al. The incidence of perioperative myocardial infarction in men undergoing noncardiac surgery. *Ann Intern Med* 1993; **118**: 504–510.
3. Hollenberg M, Mangano DT, Browner WS, London MJ, Tubau JF, Tateo IM. Predictors of postoperative myocardial ischemia in patients undergoing noncardiac surgery: The Study of Perioperative Ischemia Research Group. *JAMA* 1992; **268**: 205–209.
4. Hubbard BL, Gibbons RJ, Lapeyre AC 3rd, Zinsmeister AR, Clements IP. Identification of severe coronary artery disease using simple clinical parameters. *Arch Intern Med* 1992; **152**: 309–312.
5. Lette J, Waters D, Bernier H, Champagne P, Lassonde J, Picard M, et al. Preoperative and long-term cardiac risk assessment: Predictive value of 23 clinical descriptors, 7 multivariate scoring systems, and quantitative dipyridamole imaging in 360 patients. *Ann Surg* 1992; **216**: 192–204.
6. Mangano DT, Browner WS, Hollenberg M, London MJ, Tubau JF, Tateo IM. Association of perioperative myocardial ischemia with cardiac morbidity and mortality in men undergoing noncardiac surgery: The Study of Perioperative Ischemia Research Group. *N Engl J Med* 1990; **323**: 1781–1788.
7. Michel LA, Jamart J, Bradpiece HA, Malt RA. Prediction of risk in noncardiac operations after cardiac operations. *J Thorac Cardiovasc Surg* 1990; **100**: 595–605.
8. Goldman L, Caldera DL, Nussbaum SR, Southwick FS, Krogstad D, Murray B, et al. Multifactorial index of cardiac risk in noncardiac surgical procedures. *N Engl J Med* 1977; **297**: 845–850.
9. Wyman RM, Safian RD, Portway V, Skillman JJ, McKay RG, Baim DS. Current complications of diagnostic and therapeutic cardiac catheterization. *J Am Coll Cardiol* 1988; **12**: 1400–1406.
10. Waugh JR, Sacharias N. Arteriographic complications in the DSA era. *Radiology* 1992; **182**: 243–246.
11. Eagle KA, Berger PB, Calkins H, Chaitman BR, Ewy GA, Fleischmann KE, et al; American College of Cardiology; American Heart Association. ACC/AHA guideline update for perioperative cardiovascular evaluation for noncardiac surgery–executive summary: A report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Committee to Update the 1996 Guidelines on Perioperative Cardiovascular Evaluation for Noncardiac Surgery). *J Am Coll Cardiol* 2002; **39**: 542–553.
12. Fleisher LA, Nelson AH, Rosenbaum SH. Postoperative myocardial ischemia: Etiology of cardiac morbidity or manifestation of underlying disease? *J Clin Anesth* 1995; **7**: 97–102.
13. Landesberg G, Erel J, Anner H, Eidelman LA, Weinmann E, Luria MH, et al. Perioperative myocardial ischemia in carotid endarterectomy under cervical plexus block and prophylactic nitroglycerin infusion. *J Cardiothorac Vasc Anesth* 1993; **7**: 259–265.
14. Charlson ME, MacKenzie CR, Gold JP, Ales KL, Topkins M, Shires GT. Intraoperative blood pressure: What patterns identify patients at risk for postoperative complications? *Ann Surg* 1990; **212**: 567–580.
15. Mangano DT, Hollenberg M, Fegert G, Meyer ML, London MJ, Tubau JF, et al. Perioperative myocardial ischemia in patients undergoing noncardiac surgery–I: Incidence and severity during the 4 day perioperative period. The Study of Perioperative Ischemia (SPI) Research Group. *J Am Coll Cardiol* 1991; **17**: 843–850.
16. Eisenberg MJ, London MJ, Leung JM, Browner WS, Hollenberg M, Tubau JF, et al. Monitoring for myocardial ischemia during noncardiac surgery: A technology assessment of transesophageal echocardiography and 12-lead electrocardiography: The Study of Perioperative Ischemia Research Group. *JAMA* 1992; **268**: 210–216.
17. London MJ, Tubau JF, Wong MG, Layug E, Hollenberg M, Krupski WC, et al. The "natural history" of segmental wall motion abnormalities in patients undergoing noncardiac surgery: S.P.I. Research Group. *Anesthesiology* 1990; **73**: 644–655.
18. Grines CL, Bonow RO, Casey DE Jr, Gardner TJ, Lockhart PB, Moliterno DJ, et al; American Heart Association; American College of Cardiology; Society for Cardiovascular Angiography and Interventions; American College of Surgeons; American Dental Association; American College of Physicians. Prevention of premature discontinuation of dual antiplatelet therapy in patients with coronary artery stents: A science advisory from the American Heart Association, American College of Cardiology, Society for Cardiovascular Angiography and Interventions, American College of Surgeons, and American Dental Association, with representation from the American College of Physicians. *Circulation* 2007; **115**: 813–818.
19. Fleisher LA, Beckman JA, Brown KA, Calkins H, Chaikof E, Fleischmann KE, et al. ACC/AHA 2006 guideline update on perioperative cardiovascular evaluation for noncardiac surgery: Focused update on perioperative beta-blocker therapy: A report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Writing Committee to Update the 2002 Guidelines on Perioperative Cardiovascular Evaluation for Noncardiac Surgery) developed in collaboration with the American Society of Echocardiography, American Society of Nuclear Cardiology, Heart Rhythm Society, Society of Cardiovascular Anesthesiologists, Society for Cardiovascular Angiography and Interventions, and Society for Vascular Medicine and Biology. *J Am Coll Cardiol* 2006; **47**: 2343–2355.
20. Mangano DT, Layug EL, Wallace A, Tateo I. Effect of atenolol on mortality and cardiovascular morbidity after noncardiac surgery: Multicenter Study of Perioperative Ischemia Research Group. *N Engl J Med* 1996; **335**: 1713–1720.
21. Eagle KA, Brundage BH, Chaitman BR, Ewy GA, Fleisher LA, Hertzer NR, et al. Guidelines for perioperative cardiovascular evaluation for noncardiac surgery: Report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Committee on Perioperative Cardiovascular Evaluation for Noncardiac Surgery). *J Am Coll Cardiol* 1996; **27**: 910–948.
22. Cohen MM, Duncan PG, Tate RB. Does anesthesia contribute to operative mortality? *JAMA* 1988; **260**: 2859–2863.
23. Frank SM, Fleisher LA, Breslow MJ, Higgins MS, Olson KF, Kelly S, et al. Perioperative maintenance of normothermia reduces the incidence of morbid cardiac events: A randomized clinical trial. *JAMA* 1997; **277**: 1127–1134.
24. Kakinohana M, Odo Y, Sugahara K. Total intravenous anesthesia by propofol, fentanyl and ketamine for five cases of acute superior mesenteric artery occlusion. *Masui* 2001; **50**: 273–277 (in Japanese).
25. Wahr JA, Parks R, Boisvert D, Comunale M, Fabian J, Ramsay J, et al. Preoperative serum potassium levels and perioperative outcomes in cardiac surgery patients: Multicenter Study of Perioperative Ischemia Research Group. *JAMA* 1999; **281**: 2203–2210.
26. Yasuhara H, Naka S, Kuroda T, Wada N. Blunt thoracic and abdominal vascular trauma and organ injury caused by road traffic accident. *Eur J Vasc Endovasc Surg* 2000; **20**: 517–522.
27. Williams JS, Graff JA, Uku JM, Steinig JP. Aortic injury in vehicular trauma. *Ann Thorac Surg* 1994; **57**: 726–730.
28. Nakazawa M. Management of adult patients who have undergone surgical treatment of cardiac anomaly. *In*: Kawashima Y, Hosoda S, Sasayama S, editors. Advanced Medicine Series 12 Heart Diseases–Current Medical Practice for Heart Diseases. Tokyo: Sentan Iryo Gijutsu Kenkyusho, 2001; 206–214 (in Japanese).
29. Khairy P, Ouyang DW, Fernandes SM, Lee-Parritz A, Economy KE, Landzberg MJ. Pregnancy outcomes in women with congenital heart disease. *Circulation* 2006; **113**: 517–524.
30. Elkayam U. Pregnancy and cardiovascular disease. *In*: Braunwald E, editor. Heart disease. A textbook of cardiovascular medicine, 5th edn. Philadelphia: W.B. Saunders Company, 1997; 1843–1864.
31. Presbitero P, Rabajoli F, Somerville J. Pregnancy in patients with congenital heart disease. *Schweiz Med Wochenschr* 1995; **125**: 311–315.
32. Akagi T, Yamakawa R, Kato H. Pregnancy and childbirth after surgical treatment of congenital heart disease. *Heart Nursing* 1998; **11**: 823–827 (in Japanese).
33. Presbitero P, Somerville J, Stone S, Aruta E, Spiegelhalter D, Rabajoli F. Pregnancy in cyanotic congenital heart disease: Outcome of mother and fetus. *Circulation* 1994; **89**: 2673–2676.
34. Elkayam U, Cobb T, Gleicher N. Congenital heart disease in pregnancy. *In*: Elkayam U, Gleicher N, editors. Cardiac problem in pregnancy. Diagnosis and management of maternal and fetal disease, 2nd edn. New York: Alan R. Liss, Inc., 1900; 73–98.
35. Whittemore R, Hobbins J, Engle M. Pregnancy and its outcomes in women with and without surgical treatment in congenital heart disease. *Am J Cradiol* 1982; **50**: 641–651.
36. Connoly HM, Warnes CA. Ebstein's anomaly: Outcome of pregnancy. *J Am Coll Cardiol* 1994; **23**: 1194–1198.
37. Weiss BM, Zemp L, Seifert B, Hess OM. Outcome of pulmonary vascular disease in pregnancy: A systemic overview from 1978 through 1996. *J Am Coll Cardiol* 1998; **31**: 1650–1657.
38. Hung L, Rahimtoola SH. Prosthetic hearts valves and pregnancy. *Circulation* 2003; **107**: 1240–1246.
39. ACC/AHA guidelines for the management of patients with valvular heart disease. *J Am Coll Cardiol* 1998; **32**: 1486–1588.
40. Oakley CM. Anticoagulants in pregnancy. *Br Heart J* 1995; **74**: 107–111.

41. Elkayam U. Anticoagulation in pregnant women with prosthetic heart valves: A double jeopardy. *J Am Coll Cardiol* 1996; **27**: 1704–1706.
42. Sbarouni E, Oakley CM. Outcome of pregnancy in women with valve prostheses. *Br Heart J* 1994; **71**: 196–201.
43. Elkayam U, Bitar F. Valvular heart disease and pregnancy: Part II: Prosthetic valves. *J Am Coll Cardiol* 2005; **46**: 403–410.
44. Akagi T, Kato H. Pregnancy and childbirth: General introduction. *In*: Monma K, editor. Guideline-based clinical practice for patients with congenital heart disease. Tokyo: CHUGAI-IGAKUSHA, 2001; 25–28 (in Japanese).
45. Guidelines for the Diagnosis and Treatment of Cardiovascular Diseases (2004–2005 JCS joint working groups report). Guidelines for the clinical application of bypass grafts and the surgical techniques. *Circ J* 2006; **70**(Suppl IV): 1477–1553 (in Japanese).
46. Varadarajan P, Kapoor N, Bansal RC, Pai RG. Clinical profile and natural history of 453 nonsurgically managed patients with severe aortic stenosis. *Ann Thorac Surg* 2006; **82**: 2111–2115.
47. Bonow RO, Carabello B, de Leon AC Jr, Edmunds LH Jr, Fedderly BJ, Freed MD, et al. Guidelines for the management of patients with valvular heart disease: Executive summary: A report of American College of Cardiology/American Heart Association Task Force on Practice Guideline (Committee on Management of Patients with Valvular Heart Disease). *Circulation* 1998; **98**: 1949–1984.
48. Dajani AS, Bisno AL, Chung KJ, Durack DT, Freed M, Gerber MA, et al. Prevention of bacterial endocarditis: Recommendations by the American Heart Association. *JAMA* 1990; **264**:2919–2922.
49. Henry WL, Bonow RO, Borer JS, Ware JH, Kent KM, Redwood DR, et al. Observation on the optimum timing for operative intervention for aortic regurgitation. I: Evaluation of the results of aortic valve replacement in symptomatic patients. *Circulation* 1980; **61**: 471–483.
50. Carabello BA, Usher BW, Hendrix GH, Assey ME, Crawford FA, Leman RB. Predictors of outcome for aortic valve replacement in patients with aortic regurgitation and left ventricular dysfunction: A change in the measuring stick. *J Am Coll Cardiol* 1987; **10**: 991–997.
51. Hirota Y, Kusukawa J, Shimizu G, Kaku K, Kita Y, Saito T, et al. Optimal timing valve replacement in chronic aortic regurgitation: Analysis based on the myocardial contractility and postoperative prognosis. *J Cardiol* 1988; **18**: 747–756.
52. Hayes SN, Holmes DR Jr, Nishimura RA, Reeder GS. Palliative percutaneous aortic balloon valvuloplasty before noncardiac operations and invasive diagnostic procedures. *Mayo Clin Proc* 1989; **64**: 753–757.
53. Stein PD, Alpert JS, Copeland J, Dalen JE, Goldman S, Turpie AGG. Antithrombotic therapy in patients with mechanical and biological prosthetic heart valves. *Chest* 1992; **102**(Suppl): 445S–455S.
54. Baum VC, Barton DM, Gutgesell HP. Influence of congenital heart disease on mortality after noncardiac surgery in hospitalized children. *Pediatrics* 2000; **105**: 332–335.
55. Yamaguchi M. Postoperative management of pediatric surgical disorders associated with cardiovascular anomalies in neonate and early infancy. *Jap J Pediat Surg* 1984; **16**: 1337–1345 (in Japanese).
56. Mee RBB, Beasley SW, Auldist AW, Myers NA. Influence of congenital heart disease on management of oesophageal atresia. *Pediatr Surg Int* 1992; **7**: 90–93.
57. Satoh Y, Iwaya F, Igari T, Satokawa H, Takase S, Hoshino S, et al. Two cases of congenital cardiac anomaly associated with esophageal atresia. *Kyobu Geka* 1992; **45**: 424–427 (in Japanese).
58. Spitz L, Kiely E, Brereton RJ, Drake D. Management of esophageal atresia. *World J Surg* 1993; **17**: 296–300.
59. Hirobe S, Hayashi A, Kamagata S, Fuchimoto Y, Shimono R, Kodera A, et al. Management of esophageal atresia associated with congenital heart disease. *Jpn J Pediatr Surg* 2000; **32**: 921–927 (in Japanese).
60. Kimble RM, Harding J, Kolbe A. Additional congenital anomalies in babies with gut atresia or stenosis: When to investigate, and which investigation. *Pediatr Surg Int* 1997; **12**: 565–570.
61. Rescorla FJ, Grosfeld JL. Intestinal atresia and stenosis: Analysis of survival in 120 cases. *Surgery* 1985; **98**: 668–676.
62. Grosfeld JL, Rescorla FJ. Duodenal atresia and stenosis: Reassessment of treatment and outcome based on antenatal diagnosis, pathologic variance, and long-term follow-up. *World J Surg* 1993; **17**: 301–309.
63. Taguchi T, Uesugi T, Nagata K, Masumoto K, Suita S. Cardiac anomalies associated with congenital diaphragmatic hernia. *Jpn J Pediatr Surg* 2006; **38**: 163–169 (in Japanese).
64. Momma K, Kamiya T, Imai Y, Nagashima M, Matsuda H, Shirotani H, et al. Guidelines for standard management of patients following

65. surgical treatment of congenital heart diseases: A report of a study on "Evaluation of postoperative conditions of congenital heart diseases and establishment of criteria for postoperative management" by the MHW-Sponsored Cardiovascular Disease Study Group. *Pediatric Cardiology and Cardiac Surgery* 1994; **9**: 589–599 (in Japanese).
65. Pitt MP, Bonser RS. The natural history of thoracic aortic aneurysm disease: An overview. *J Card Surg* 1997; **12**(2 Suppl): 270–278.
66. Tabayashi K, Suzuki Y, Niibori N, Ito Y, Sekino Y, Togo T, et al. Treatment of aneurysms of the aortic arch. *Kyobu Geka* 1991; **44**: 531–536 (in Japanese).
67. Takamiya M, Kuribayashi S, Hamada S, Iino M. Recent progress of diagnostic imaging for aortic dissection. *Nippon Geka Gakkai Zasshi* 1996; **97**: 884–889 (in Japanese).
68. Prince MR. Gadolinium-enhanced MR aortography. *Radiology* 1994; **191**: 155–164.
69. Landesberg G, Mosseri M, Wolf YG, Bocher M, Basevitch A, Rudis E, et al. Preoperative thallium scanning, selective coronary revascularization and long-term survival after major vascular surgery. *Circulation* 2003; **108**: 177–183.
70. Breen P, Lee JW, Pomposelli F, Park KW. Timing of high-risk vascular surgery following coronary artery bypass surgery: A 10-year experience from an academic medical centre. *Anaesthesia* 2004; **59**: 422–427.
71. The fifth report of the Joint National Committee on Detection, Evaluation, and Treatment of High Blood Pressure (JNCV). *Arch Intern Med* 1993; **153**: 154–183.
72. Islamoğlu F, Atay Y, Can L, Kara E, Ozbaran M, Yüksel M, et al. Diagnosis and treatment of concomitant aortic and coronary disease: A retrospective study and brief review. *Tex Heart Inst J* 1999; **26**: 182–188.
73. Hafez KS, El Fettouh HA, Novick AC, Ouriel K. Management of synchronous renal neoplasm and abdominal aortic aneurysm. *J Vasc Surg* 2000; **32**: 1102–1110.
74. Morris HL, da Silva AF. Co-existing abdominal aortic aneurysm and intra-abdominal malignancy: Reflections on the order of treatment. *Br J Surg* 1998; **85**: 1185–1190.
75. EVAR trail participants. Endovascular aneurysm repair and outcome in patients unfit for open repair of abdominal aortic aneurysm (EVAR trail 2): Randomised controlled trial. *Lancet* 2005; **365**: 2187–2192.
76. Coselli JS, LeMaire SA, Buket S. Marfan syndrome: The variability and outcome of operative management. *J Vasc Surg* 1995; **21**: 432–443.
77. Groenink M, Lohuis TA, Tijssen JG, Naeff MS, Hennekam RC, van der Wall EE, et al. Survival and complication free survival in Marfan's syndrome: Implications of current guidelines. *Heart* 1999; **82**: 499–504.
78. Lord JW Jr, Rossi G, Daliana M, Drago JR, Schwartz AM. Unsuspected abdominal aortic aneurysms as the cause of peripheral arterial occlusive disease. *Ann Surg* 1973; **177**: 767–771.
79. Nemir P Jr, Micozzi MS. Combined aneurysmal and occlusive arterial disease. *Circulation* 1977; **56**(Suppl 2): 169–170.
80. Baxter BT, McGee GS, Flinn WR, McCarthy WJ, Pearce WH, Yao JS. Distal embolization as a presenting symptom of aortic aneurysms. *Am J Surg* 1990; **160**: 197–201.
81. Beneficial effect of carotid endarterectomy in symptomatic patients with high-grade carotid stenosis. North American Symptomatic Carotid Endarterectomy Trial Collaborators. *N Engl J Med* 1991; **325**: 445–453.
82. Randomised trial of endarterectomy for recently symptomatic carotid stenosis: Final results of the MRC European Carotid Surgery Trial (ECST). *Lancet* 1998; **351**: 1379–1387.
83. Norgren L, Hiatt WR, Dormandy JA, Nehler MR, Harris KA, Fowkes FG. Inter-Society Consensus for the Management of Peripheral Arterial Disease (TASC II). *J Vasc Surg* 2007; **45**(Suppl S): S5–S67.
84. Rubin LJ; American College of Chest Physicians. Diagnosis and management of pulmonary hypertension: ACCP evidence-based clinical practice guideline. *Chest* 2004; **126**(1 Suppl): 7S–10S.
85. Richardson P, McKenna W, Bristow M, Maisch B, Mautner B, O'Connell J, et al. Report of the 1995 World Health Organization/International Society and Federation of Cardiology Task Force on the Definition and Classification of cardiomyopathies. *Circulation* 1996; **93**: 841–842.
86. Kawai H, Yokoyama M. Dilated cardiomyopathy: Diagnostic criteria/clinical findings/chest X-ray, phonocardiogram, electrocardiography/echocardiography, Doppler method. *In*: Matsumori A, editor. Cardiomyopathy. Tokyo: MEDICAL VIEW CO. LTD, 2000; 106–113 (in Japanese).
87. O'Kelly B, Browner WS, Massie B, Tubau J, Ngo L, Mangano DT.

Ventricular arrhythmias in patients undergoing noncardiac surgery: The Study of Perioperative Ischemia Research Group. *JAMA* 1992; **268:** 217–221.

88. Gibson SC, Payne CJ, Byrne DS, Berry C, Dargie HJ, Kingsmore DB. B-type natriuretic peptide predicts cardiac morbidity and mortality after major surgery. *Br J Surg* 2007; **94:** 903–909.

Appendix

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Evaluation of the Prospective Observation of erythropoietin-administration for the treatment of Acute Myocardial Infarction (EPO/AMI-1) Study

鳥羽　健　加藤　公則　小澤　拓也　相澤　義房

Evaluation of the Prospective Observation of erythropoietin-administration for the treatment of Acute Myocardial Infarction (EPO/AMI-1) Study

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Key words　急性心筋梗塞，エリスロポエチン，心不全予防

1. はじめに

エリスロポエチン（EPO）は熊本大学の宮家博士によって純化精製された赤血球造血ホルモンで[1]，腎性貧血治療薬として 20 年以上の歴史をもつ．前世紀末に，中枢神経系にEPO分泌細胞とEPO受容体（EPOR）陽性細胞の両者が存在し，各種の中枢神経障害モデル動物に対し外来性に投与されたEPO製剤が中枢神経保護的に作用することが示された[2]．その後，心臓・腎臓その他の臓器にもEPORが発現していることがわかり，家兎の心臓虚血再環流モデルに対して外来性に投与されたEPO製剤が心筋保護的に作用することが示され[3]，多数の追試によって確認された．これにより，日欧米で心筋梗塞患者に対するEPO投与による臨床試験実施の気運が高まった．

急性心筋梗塞（AMI）に対する標準的治療法として経皮的冠動脈形成術（PCI）や冠動脈バイパス術（CABG）が行われるようになって救命率が著しく改善したが，術後慢性期の心機能低下を来す症例が少なからず存在する．このような症例に対し，標準的治療法に何らかの薬物療法を追加することで慢性心不全の合併を軽減できれば，患者にとっての生活の質の改善や社会における人的資源の向上，さらには医療費や社会保障費の削減に繋がる．

新潟大学医歯学総合病院第一内科
Series：Clinical Study from Japan and its Reflections；Evaluation of the Prospective Observation of erythropoietin-administration for the treatment of Acute Myocardial Infarction（EPO/AMI-1）Study.
Ken Toba, Kiminori Kato, Takuya Ozawa and Yoshifusa Aizawa : First Department of Internal Medicine, Niigata University Medical and Dental Hospital, Japan.
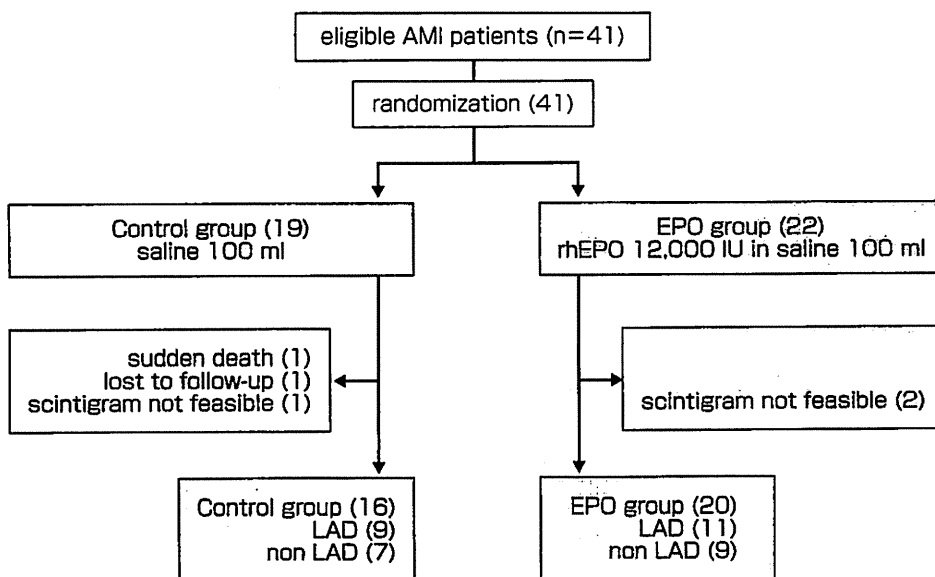
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        eligible AMI patients (n=41)

            randomization (41)

  Control group (19)          EPO group (22)
  saline 100 ml               rhEPO 12,000 IU in saline 100 ml

  sudden death (1)
  lost to follow-up (1)       scintigram not feasible (2)
  scintigram not feasible (1)

  Control group (16)          EPO group (20)
  LAD (9)                     LAD (11)
  non LAD (7)                 non LAD (9)
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図1. EPO/AMI-1 Studyに登録された症例 （文献4より引用）

2. EPO/AMI-1 Study

われわれは2005年に臨床試験を計画し，患者登録を開始した．対象は発症後24時間以内にPCIに成功した1枝病変のST上昇型AMI患者で，説明と同意の後にEPO投与群と生食投与群に無作為割り付けした（オープンラベル）．EPOは12,000国際単位（IU）のエポエチンベータ製剤を生食100 mLに希釈して1時間かけて静脈内に投与した．2005年12月から2008年8月までに41例の患者が登録され，そのうち36例が解析可能であった（図1）[4]．主要評価項目は急性期と6カ月後に実施したTc-MIBI SPECTによる左室機能の改善である（図2）.

コントロール群では6カ月の間に左室駆出率（LVEF）が$4.1 \pm 12.1\%$ 自然回復（NS）したのに対し，EPO投与群では$7.6 \pm 12.5\%$ 回復（$p < 0.05$）したが，回復の程度（急性期と6カ月後のLVEFの差）は両群間で有意差が得られなかった．但し図には示さなかったが，心筋梗塞部位の壁運

動の指標とされる局所駆出率の6カ月間の改善についてはコントロール群に比しEPO投与群で有意に良好であった（$p < 0.01$）．解剖学的理由から心機能におよぼす影響の大きい左冠動脈前下行枝（LAD）に病変のある患者では急性期のLVEFが50%未満の症例が多く，EPO投与によるLVEFの改善が著しかった．

Tc-MIBIシンチの情報から心筋梗塞領域を算出するためには，従来から左室を極座標に展開（Bull's eye表示）して血流低下領域をおおまかに見積もる方法が用いられてきた．われわれはTc-MIBIシンチの短軸切片を心尖部から心基部まで積分し，リアルな血流低下領域容積を計算する新しいソフトを共同開発し，今回の臨床試験に初めて用いた（図3）[5]．コントロール群では6カ月間で$0.9 \pm 12.0 \, cm^3$とほとんど変化がなかったのに対し，EPO投与群では$12.5 \pm 19.7 \, cm^3$の縮小（$p < 0.05$）が観察され，また両群間で有意差が見られた（$p < 0.05$）.

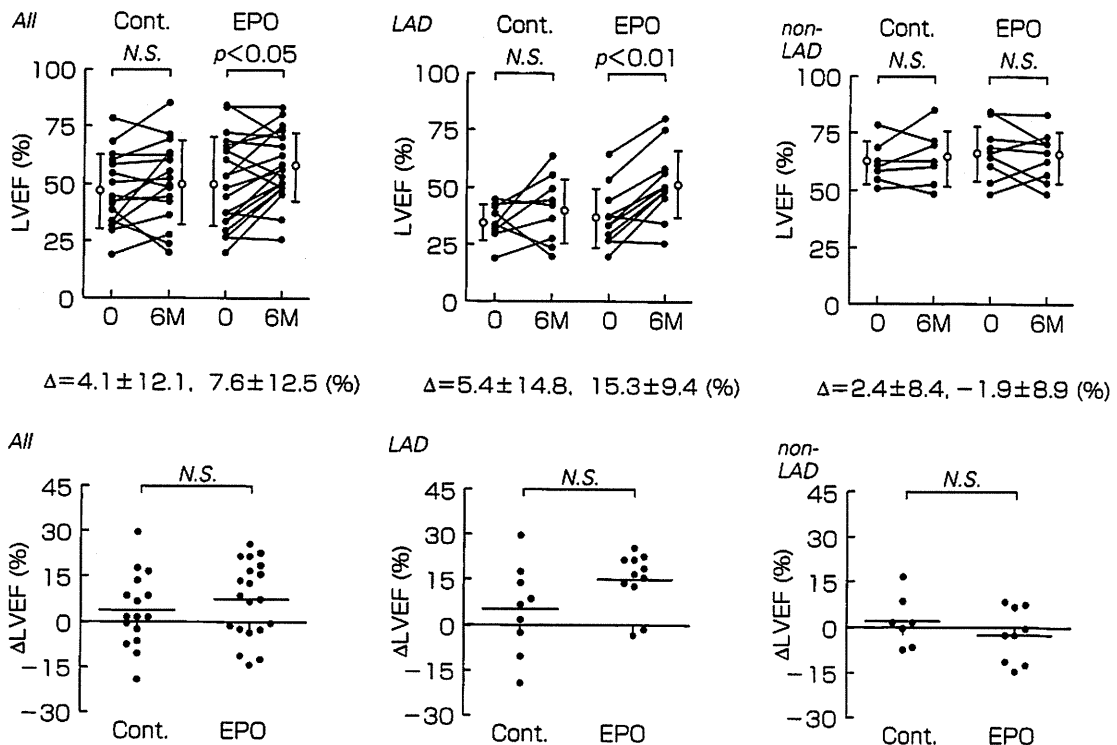これらの結果から，EPOの投与は標準的治療法に対する上乗せ効果として慢性期の心機能を

図2. 急性期と6ヶ月後の左室駆出率（Tc-MIBIシンチ，Wilcoxon test）（文献4より引用）

改善する可能性が推測され，より大規模な臨床試験を行いエビデンスを示す必要が生じた．

3. 舞台裏

21世紀早々，心・血管系の再生医学研究とその臨床応用が話題となりはじめ，新潟も遅れてはならじという教授の指示により，血液内科と循環器内科の共同で再生医療チームを立ち上げた．当時関西医大におられた松原弘明先生の指導のもとに自家骨髄細胞の異所性移植による血管再生治療を開始した．骨髄細胞による血管再生の機序を研究するうちに，未熟赤芽球の重要な役割に気付き[6]，また偶然EPOの心血管系に対する作用を見いだした．これをきっかけに未熟赤芽球を用いた新しい血管新生治療の開発や[7]，中外製薬との共同研究によるEPO誘導体に関す

るいくつかの特許につながった．一方，米国のAnthony Ceramiのグループは既に中枢神経系や心臓に対するEPOの作用を見いだして特許に結びつけており，この領域をリードしていた．

Duke大学のグループから2003年のJ. Clin Invest誌に，家兎の虚血再環流モデルでEPO投与が心筋梗塞を改善するという論文発表があり[3]，注目を集めた．2005年には既に大阪大学の南野哲男先生のグループが心筋梗塞に対するEPOの治験を始めるらしいといううわさがあった．そこで我々と鈴木洋先生（昭和大学），横山真一郎先生（日本大学），小林直彦先生（獨協大学）が中心となって研究会を立ち上げ，2005年中に4回の会合を開いて心筋梗塞に対するEPOの治験についての研究計画策定を行い，各施設の倫理委員会やIRBの承認を受けて2005年末から患者登録を開始した．
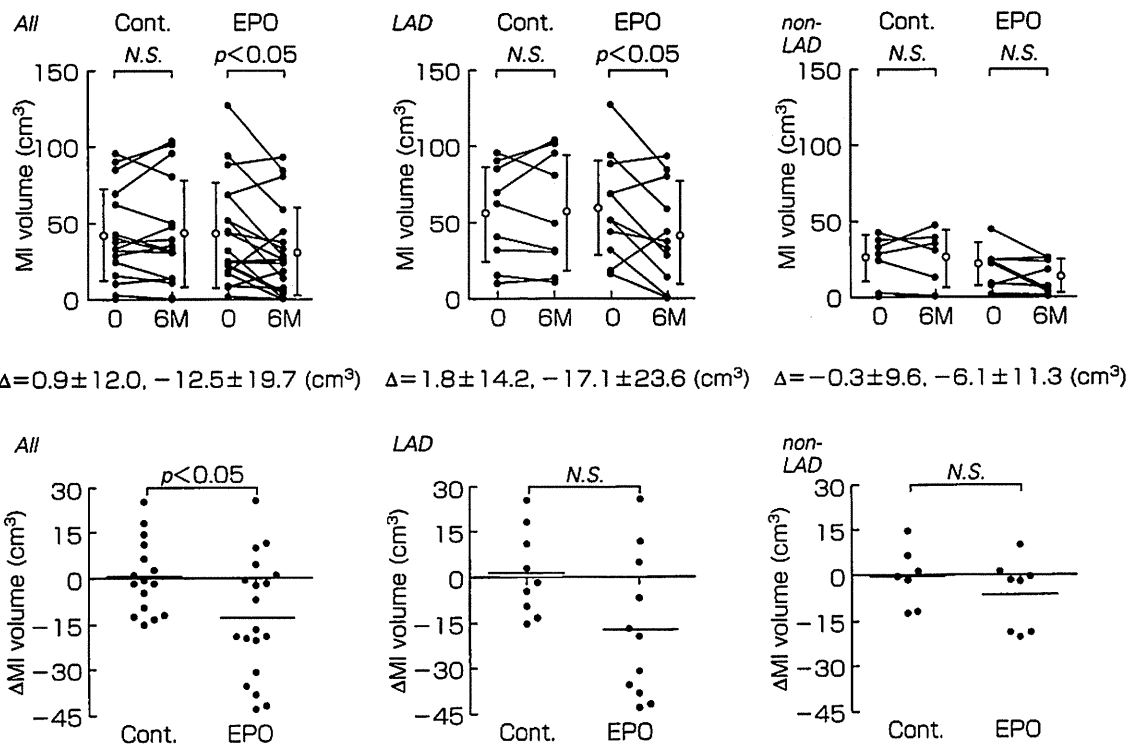
図3. 急性期と6ヶ月後の血流低下領域容積（Tc-MIBIシンチ，Wilcoxon test）（文献5より引用）

当時はEPOの投与経路・量・日数などについて参考となる臨床試験の報告がなく，手探り状態であった．当初は6,000 IUの3日間投与という考えと，超急性期の心筋アポトーシス進行時に心筋保護をすれば良いという12,000 IUの1回投与の2つの考えが出た．腎性貧血患者に対するEPOの副作用として高血圧や脳心血管イベントの誘発など，本研究の目的とは相容れない有害事象が知られていた．鉄欠乏性貧血患者の高EPO血症による血小板増多は日常よく経験するところであるが，EPOにはトロンボポエチン様作用があり，血小板増加および成熟血小板の機能亢進誘導が知られている．そのため安全性を最重視し，腎性貧血に使われていた最大量の12,000 IUを採用した．また複数回の投与は心筋梗塞患者には好ましくない多血症その他の効果を示すことが分かっていたので，造血作用を起こさな

い1回のみの投与とした．この投与レジメがAMI患者に有効性を示すことは当初よりあまり期待はしていなかったが，病変部位に病的および医原的な血管内皮障害があり血小板血栓のできやすい状態になっているPCI後の患者に対してEPOが安全に投与できれば，その後にdose-escalation studyを組むことが可能となると考えた．

もう一つの大きなテーマは主要評価項目の設定である．目的である心機能の評価にMRIと心筋シンチのいずれを採用するか迷ったが，医療現場で需要の多いMRIを救急のAMI患者に優先的に使用することの困難があり，血流低下領域の定量と同時に心機能や局所壁運動の評価も行える心筋シンチを採用することになった．6カ月後の効果判定時には薬剤負荷心筋シンチを行うこととした．パイロット研究なので盲検とはせずにオープンラベルで行い，封筒法による無作

表. 各国の主な臨床試験

		対象	症例数	国際単位×日数	評価	判定
REVIVAL-3	ミュンヘン	多枝病変を含む	138例	33,000×3	MRI，梗塞サイズ，6カ月後	群間有意差なし
					脳心血管イベント	増加させる可能性あり
HEBE-Ⅲ	オランダ	多枝病変を含む	448例	60,000×1	Tc-シンチ，LVEF，6週後	群間有意差なし
					6週間の心血管イベント	群間有意差あり（改善）
					6週後のNT-proBNP	群間有意差あり（改善）
EPOC-AMI	京都	多枝病変を含む	35例	6,000×3	Tc-シンチ，LVEF，6カ月後	群間有意差なし？但しEPO群内でLVEF改善
					Tc-シンチ，梗塞サイズ，6カ月後	群間有意差なし？但しEPO群内で梗塞サイズ縮小
EPO/AMI-1	本研究	1枝病変のみ	36例	12,000×1	Tc-シンチ，LVEF，6カ月後	群間有意差なし但しEPO群内でLVEF改善
					Tc-シンチ，rEF，6カ月後	群間有意差あり（改善）
					Tc-シンチ，梗塞サイズ，6カ月後	群間有意差あり（改善）
EPAMINODAS	ローマ	多枝病変を含む	102例	6,000×3 12,000×3	梗塞サイズ（CK-MB）MRI，LVEF，etc	ongoing
REVEAL	米国	多枝病変を含む	210例	15,000×1 30,000×1 60,000×1	MRI，梗塞サイズ，3カ月後	ongoing

為割り付けを採用した．評価にバイアスがかかることを避ける目的で，主要評価項目の資源であるシンチのデータ媒体を一旦センターに集積し，患者情報を知らせていない放射線専門医にblindでの解析を依頼することで解析結果に一定の質を保証した．

症例登録に対する奨励金の支給なし，会合は東京周辺で開催される学会のついで，参加者の手弁当が原則で経費は事実上0円であった．2008年2月の第14回の研究会からは南野哲男先生にもご参加いただき，後続の大規模検証研究のための布石となった．当初より安全性を重視した試験であり，データ解析で有意差が出たと聞いたときには我が耳を疑った．さっそく2009年3月の日本循環器学会総会Late Breaking Clinical Trialsで鈴木洋先生が発表した．

4. 他の臨床研究

現段階では，AMIに対するEPO投与の臨床試験に関する報告が4つあるが，決定的に有効であるというエビデンスはない（表）．EPOの投与量や投与方法，および評価方法の違いが結論を左右しているようにみえる．ドイツのREVIVAL-3 studyでは33,000 IU×3日間という大量連日投与を行い，6カ月後のMRIで梗塞サイズの有意な改善を認めていない[8]．中間解析でEPO投与による脳心血管イベントの増加の可能性について注意を喚起していた．オランダのHEBE-Ⅲ studyでは，60,000 IU×1回のボラス投与を行っている[9]．6週間後のLVEFで有意な改善を認めなかったが，NT-proBNPの有意な低下および心血管イベントの有意な減少が見られた．Voorsは欧州心臓病学会2010の報告で，彼らの研究が不十分な結果に

終わった原因として，評価項目および評価時期の問題である可能性に言及している．本邦のEPOC-AMI研究では6,000 IU×3日間の投与を行い，われわれと同様に6カ月後のLVEFおよび心筋梗塞サイズでEPO群に有意な改善を認めたが，両群間の有意差については不明である[10]．我々の結果も合わせて考えると，EPO大量投与より通常量投与の方がより有効である可能性が示唆されるが，大規模スタディーを行うまでは結論を出せない．現在進行中の研究にEPAMINODAS study[11]とREVEAL study[12]があり，結果に興味がもたれる．

ラットの虚血再環流実験ではEPOに至適投与量が存在し，大量のEPO投与では効果が減弱する可能性がある[13]．ヒトにEPO製剤を静脈内投与した場合の血中濃度の推移を見ると[14]，体重60 kgの成人に換算して9,000〜18,000 IUの投与で約半日間は1 IU/ml以上の血中濃度が維持されることがわかり，急性心筋梗塞再環流後のGolden hour内には十分な量であることが推測される．総合的に判断すると，EPO/AMI-1で仮に採用した投与量が，偶然に血小板刺激による心血管イベントを誘発することなしに心筋保護作用を示す至適投与量であったのかもしれない．少なくとも欧州の臨床試験のように腎性貧血の治療に用いる量の数倍から数十倍の量を投与しなくてはいけない理由は思いつかない．

5. 今後の展望

前述のとおりEPO/AMI-1の結果は有望ではあるがエビデンスとは言えない．もしEPO投与が心機能を本当に改善するのであれば，それを多数例の試験によって科学的に実証することで，インターベンション後の標準的薬物療法として確立し，医療に貢献することは臨床医の義務である．多施設共同の大規模臨床研究EPO/AMI-2 studyを計画した．本計画の策定には大阪大学循環器内科の小室一成先生・南野哲男先生・彦惣俊吾先生および鳥羽が中心となり，大阪大学医学部附属病院の未来医療センターが治験の拠点として全面的に協力してくれた．本研究は日本循環器学会のTranslational Research支援事業および厚生労働科学研究費補助金（医療技術実用化総合研究事業）に採択され，厚生労働省の高度医療評価制度を利用して2011年度に開始されることとなった．以下に概略を記載する．

【対象症例】PCIに成功したSTEMI症例で，急性期に低心機能（左室駆出率50％未満）を呈する患者．観察期間の6カ月以内にさらなる血行再建術が必要な症例を除外する．実質的に1枝病変患者が適応となり，その多くがLADの1枝病変となる．

【割り付け】プラセボ，EPO 6,000 IU，EPO 12,000 IUのそれぞれ静注．各200症例，3群で合計600症例．二重盲検．

【評価項目】急性期および6カ月後のTc-MIBIシンチによる左室機能および心筋梗塞容積．生存率，心血管事故率．NT-ProBNP値．

文 献

1) Miyake T, et al : Purification of human erythropoietin. J Biol Chem 252 : 5558-5564, 1977.

2) Cerami A : Beyond erythropoiesis : novel applications for recombinant human erythropoietin. Semin Hematol 38 (3 Suppl 7) : 33-39, 2001.

3) Parsa CJ, et al : A novel protective effect of erythropoietin in the infarcted heart. J Clin Invest 112 : 999-1007, 2003.

4) Ozawa T, et al : Single-dose intravenous administration of recombinant human erythropoietin is a promising treatment for patients with acute myocardial infarction—randomized controlled pilot trial of EPO/AMI-1 study—. Circ J 74 : 1415-1423, 2010.

5) Yoshimura N, et al : A novel program to accurately quantify infarction volume by (99m) Tc MIBI SPECT, and its application for re-analyzing the effect of erythropoietin administration in patients with acute myocardial

infarction. Circ J 74 : 2741-2743, 2010.

6) Ozawa T, et al : Erythroid cells play essential roles in angiogenesis by bone marrow cell implantation. J Mol Cell Cardiol 40 : 629-638, 2006.

7) Oda M, et al : Establishment of culturing system for ex-vivo expansion of angiogenic immature erythroid cells, and its application for treatment of patients with chronic severe lower limb ischemia. J Mol Cell Cardiol 49 : 347-353, 2010.

8) Ott I, et al : Erythropoietin in patients with acute ST-segment elevation myocardial infarction undergoing primary percutaneous coronary intervention:a randomized, double-blind trial. Circ Cardiovasc Interv 3 : 408-413, 2010.

9) Voors AA, et al : A single dose of erythropoietin in ST-elevation myocardial infarction. Eur Heart J 31 : 2593-2600, 2010.

10) Taniguchi N, et al : Erythropoietin prevention trial of coronary restenosis and cardiac remodeling after ST-elevated acute myocardial infarction (EPOC-AMI) : a pilot, randomized, placebo-controlled study. Circ J 74 : 2365-2371, 2010.

11) Andreotti F, et al : Update on phase II studies of erythropoietin in acute myocardial infarction. Rationale and design of Exogenous erythropoietin in Acute Myocardial Infarction : New Outlook aNd Dose Association Study (EPAMINONDAS). J Thromb Thrombolysis 28 : 489-495, 2009.

12) www.ClinicalTrial.gov Identifier : NCT 00378352.

13) Baker JE, et al : Darbepoetin alfa protects the rat heart against infarction : dose-response, phase of action, and mechanisms. J Cardiovasc Pharmacol 49 : 337-345, 2007.

14) Remakrishnan R, et al : Pharmacokinetic and pharmacodynamic modeling of recombinant human erythropoietin after single and multiple doses in healthy volunteers. J Clin Pharmacol 44 : 991-1002, 2004.

NANOMEDICINE
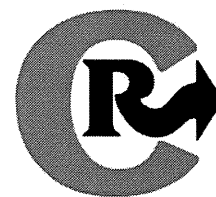
Amelioration of cerebral ischemia–reperfusion injury based on liposomal drug delivery system with asialo-erythropoietin

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ABSTRACT

Cerebral ischemia–reperfusion (I/R) injury induces secondary cerebral damage. As drugs for treating this type of injury have shown poor efficacy and adverse side effects in clinical trials, a novel therapeutic strategy has been long awaited. In this study, we focused on the disruption of the blood–brain barrier after stroke, and applied a liposomal drug delivery system (DDS) designed to enhance the pharmacological effect of the neuroprotectant and to avoid side effects. PEGylated liposomes were injected at varying time after the start of reperfusion in transient middle cerebral artery occlusion (t-MCAO) model rats. The results showed PEGylated liposomes accumulated in the ischemic hemisphere at an early stage after reperfusion and were retained in the lesion for at least 24 h after injection. We also investigated the effectiveness of asialo-erythropoietin (AEPO)-modified PEGylated liposomes (AEPO-liposomes) in treating the cerebral I/R injury. AEPO-liposome treatment significantly reduced TTC-defined cerebral legion following cerebral I/R injury, and ameliorated motor function compared with vehicle and AEPO treatment. In conclusion, these results indicate that AEPO-liposomes are a promising liposomal formulation for protecting the brain from I/R injury, and that this liposomal DDS has potential as a novel strategy for the treatment of cerebral I/R injury.

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1. Introduction

Cerebral vascular permeability increases after cerebral stroke, disrupting the integrity of the blood–brain barrier (BBB) via several mediators [1,2]. Although BBB disruption causes cerebral edema, resulting in neurological deficits [3], this increase in cerebral vascular permeability also permits drugs, which cannot penetrate the BBB under the normal condition, to accumulate in the brain parenchyma. A therapeutic strategy focused on increasing cerebral vascular permeability has succeeded in the treatment of some diseases [4]. Nanoparticles such as liposomes are used as a drug carrier for such a therapeutic strategy. They pass through the intercellular space between vascular endothelial cells and accumulate in the tissue owing to the enhanced permeability and retention (EPR) effect [5–9]. In addition, polyethylene glycol-modified liposomes (PEGylated liposomes) possess a long circulating property in the bloodstream by avoiding interaction with opsonins and the cells of the mononuclear phagocytic system [10]. PEGylated liposomes have been used to increase drug stability, safety, and bioavailability in humans.

Cerebral ischemia/reperfusion (I/R) injury is a secondary injury caused by oxidative stresses and inflammatory responses after recovery from cerebral ischemia [11,12], and this injury worsens the pathological condition. Although the results of many experimental and clinical studies have been published, effective therapeutic strategies for the treatment of acute stroke have not yet been achieved, due to poor efficacy and to adverse side effects in clinical trials [13–16]. Therefore, an increase in drug efficacy with fewer side effects is highly desirable for achieving neuroprotection in stroke patients.

In recent years, erythropoietin (EPO) has been shown to be cytoprotective in the brain [17,18]. Indeed, EPO binds to EPO receptor (EPOR) expressed on neuronal cells and reduces brain damage by activating MAPK and PI3K/Akt, and by increasing the expression of Bcl-x, resulting in the improvement of cerebral stroke outcome in permanent or transient cerebral ischemic animal models [18–21]. Moreover, EPOR-knockout mice show increased sensitivity to hypoxia and apoptosis of brain cells [22]. These data indicate that EPO acts as one of the important factors for neuroprotection and neurodevelopment after an ischemic event. However, multiple dosing with EPO might worsen the cerebral injury after a stroke; because the EPO-induced increase in the hematocrit may possibly induce thrombotic complications. Asialo-EPO (AEPO) is a metabolite of EPO that has no hematopoietic effect [23]. AEPO binds to the EPOR more strongly than does EPO because of the net positive charge afforded

by desialylation, resulting in a strong cytoprotective effect [24,25]. Although AEPO might be expected to be an effective agent for the treatment of cerebral I/R injury, it shows low accumulation in the brain parenchyma because of its short half-life. Therefore, some method of increasing the accumulation of AEPO at the site on a cerebral injury is a potential therapeutic strategy for cerebral I/R injury.

In this study, we investigated whether a liposomal DDS could be applied as a new strategy for the treatment of cerebral I/R injury. In addition, we developed AEPO-modified PEGylated liposomes (AEPO-liposomes) as a neuroprotective agent and examined the therapeutic efficacy of AEPO-liposomes in model rats with transient cerebral ischemia.

2. Materials and methods

2.1. Animal

Male Wistar rats (170–210 g) were purchased from Japan SLC, Inc. (Shizuoka, Japan). The animals were cared for according to the Animal Facility Guidelines of the University of Shizuoka. All animal procedures were approved by the Animal and Ethics Review Committee of the University of Shizuoka.

2.2. Transient middle cerebral artery occlusion model rats

Transient middle cerebral artery occlusion (t-MCAO) model rats were prepared as described previously [26]. In brief, anesthesia was induced with 3% isoflurane and maintained with 1.5% isoflurane during cerebral stroke surgery. Rectal temperature was maintained at 37 °C with a heating pad. After a median incision of the neck skin, the right carotid artery, external carotid artery, and internal carotid artery (ICA) were isolated with careful conservation of the vagal nerve. A 4-0 monofilament nylon filament coated with silicon was introduced into the right ICA and advanced to the origin of the MCA to occlude it. Silk thread was used for ligation to keep the filament at the site of insertion into the MCA. After the operation, the neck was closed and anesthesia was discontinued. MCAO was performed for 1 h. Success of the surgery was judged by the appearance of hemiparesis. Reperfusion was performed by withdrawing the filament about 10 mm at 1 h after the occlusion under isoflurane anesthesia.

2.3. Preparation of PEGylated liposomes

PEGylated liposomes composed of distearoylphosphatidylcholine (DSPC), cholesterol, and distearoylphosphatidylethanolamine (DSPE)-PEG (M.W. of PEG was 2000) (20/10/1 as molar ratio) were prepared as follows: lipids dissolved in chloroform were evaporated to form a thin lipid film by using a rotary evaporator. The lipid film was dried for at least 1 h under reduced pressure. The dried lipid film was hydrated with PBS (pH 7.4). The liposome solution was freeze-thawed for 3 cycles with liquid nitrogen and then sonicated for 15 min at 65 °C. Finally, the particle size of liposomes was adjusted by extrusion through 100 nm-pore size polycarbonate filters (Nucleopore, Cambridge, MA, USA). For some experiments, DiI-C$_{18}$ (Molecular Probes Inc., Eugene, OR, USA) or [^{3}H]cholesteryl hexadecyl ether (Perkin Elmer, Boston, MA) was mixed with initial lipid solution for labeling the liposomes.

2.4. Cerebral distribution of PEGylated liposomes

PEGylated liposomes were fluorescently labeled with DiI-C18. 10-mM DiI-labeled PEGylated liposomes (0.5 mL) were intravenously injected into the t-MCAO model rats at 0, 1, 3, 6 or 24 h of reperfusion. Their brains were dissected at 1 h after the injection and sliced into 2-mm thick coronal sections with a rat brain slicer (Muromachi Kikai, Tokyo, Japan). All sections were put in glass slides, and the fluorescence of DiI

was measured with an in vivo imaging system (IVIS, Xenogen Corp., Alameda, CA).

2.5. Preparation of AEPO-liposomes

Distearoylphosphatidylethanolamine (DSPE)-PEG-N-hydroxysuccinimide (NHS) (0.145 mg) dissolved in 480 μL of borate buffer (pH 8.4) was mixed with 20 μL of AEPO solution (0.9 mg/mL in PBS), and the mixture was incubated for 1 day at room temperature to prepare DSPE-PEG-AEPO conjugates. A 20-mM solution of PEGylated liposomes was prepared, and then 1 mL of the liposomes was incubated with 0.5 mL of the DSPE-PEG-AEPO conjugates for 15 min at 65 °C. The AEPO-modified liposomes (AEPO-liposomes) were purified by gel filtration with Sepharose™ 4 Fast Flow (Amersham Biosciences, Sweden). The AEPO concentration of AEPO-liposomes was measured by HPLC.

2.6. Cell culture

Pheochromocytoma cells (PC12 cells, ECACC, UK) were cultured in high-glucose DME medium (WAKO, Osaka, Japan) supplemented with streptomycin (100 μg/ml), penicillin (100 units/ml), heat-inactivated 5% fetal bovine serum (FBS, Japan Bioserum, Tokyo, Japan), and 10% horse serum (HS, MP Biomedicals, Solon, OH, USA) at 37 °C in a humidified chamber with 5% CO$_2$.

The PC12 cells were plated on poly-D-lysine-coated 24-well plates for the MTT assay. These cells were caused to differentiate by adding nerve growth factor (NGF) at 100 ng/ml to the DME medium containing 0.5% HS at a 48-h interval. Five days after NGF treatment, these cells were used for subsequent experiments.

2.7. Cell viability assays

Differentiated PC12 cells were treated with AEPO-liposomes (0.01, 0.1 or 1.0 nmol/L as AEPO dose) or AEPO (0.1 nmol/L) for 5 days at a 48-h interval. The number of viable cells was measured by use of TetraColor™ One (Seikagaku, Tokyo, Japan). Briefly, TetraColor™ One solution was added to each well, and the cells were then incubated at 37 °C for 3 h in a humidified atmosphere containing 5% CO$_2$. Absorbance at 450 nm was measured by using a Tecan Infinite M200 microplate reader (Tecan, Männedorf, Switzerland)

2.8. Biodistribution of AEPO-liposomes

For determination of the biodistribution of AEPO-liposomes, AEPO was radiolabeled with ^{125}I. Briefly, IODO-BEADS® Iodination Reagent (Pierce, Rockford, IL) was added to a Na^{125}I solution (1 mCi, 890 μL), and the mixture was incubated for 5 min. AEPO solution (110 μL) was then added to the reacted solution, and incubation conducted for 15 min. For removal of excess Na^{125}I or unincorporated ^{125}I, the mixture was applied to a Zeba™ Desalt Spin Column (Pierce, Rockford, IL) and centrifuged at 1000×g for 2 min.

^{125}I-labeled AEPO-liposomes were intravenously injected into the t-MCAO rats just after the start of reperfusion. At 3 and 24 h of reperfusion, the rats were sacrificed, and the blood was collected. Then the brain, heart, lung, liver, spleen, kidney, thyroid, and femur were removed and weighed. The radioactivities of organs were measured by using a gamma counter (Aloka, Tokyo, Japan).

2.9. TUNEL staining

Brains of t-MCAO model rats were dissected at 24 h after the injection of PBS or AEPO-liposomes (8 μg/kg as AEPO dose), embedded in OCT compound (Sakura Finetek, Torrance, USA), and then frozen in dry ice/ethanol. Frozen sections (10 μm) were prepared by using a cryostatic microtome (HM 505E, Microm, Walldorf, Germany) and were stained with TUNEL reagents supplied in an ApopTag® Plus Fluorescein

195 *in situ* Apoptosis Detection Kit (Chemicon International, Inc, USA), as
196 described below. For fixation of the sections, they were incubated in
197 4% paraformaldehyde for 15 min at room temperature, and in ethanol/
198 acetic acid (2:1) solution for 5 min at −20 °C. DNA strand breaks were
199 labeled with the digoxigenin-conjugated terminal deoxynucleotidyl
200 transferase enzyme by incubation for 1 h at 37 °C. Then, the sections
201 were incubated in anti-digoxigenin-fluorescein solution for 30 min at
202 room temperature. Finally, the sections were mounted with Perma
203 Fluor Aqueous Mounting Medium (Thermo Shandon, Pittsburgh, PA,
204 USA) included in DAPI solution (1.0 μg/mL) and observed for fluores-
205 cence with a microscopic LSM system (Carl Zeiss, Co., Ltd., Germany).

206 *2.10. Therapeutic experiment*

207 PBS, AEPO (8 μg/kg), AEPO-liposomes (8 μg/kg as AEPO dose) or
208 PEGylated liposomes were intravenously injected into t-MCAO rats im-
209 mediately after the start of reperfusion. The volume of damaged area,
210 the degree of brain swelling, and the functional outcome of rats were
211 assessed at 24 h of reperfusion. For investigation of the functional out-
212 come, the rats underwent a 21-point neurological score analysis prior
213 to dissection of the brain, as described previously [27]. Then their brains
214 were dissected, and the blood was collected to assess the hematopoietic
215 effect of AEPO. The brains were sliced into 2-mm-thick coronal sections
216 by using a rat brain slicer (Muromachi Kikai, Tokyo, Japan) and stained
217 with 2, 3, 5-triphenyltetrazolium chloride (TTC, Wako Pure Chemical
218 Ind. Ltd., Tokyo, Japan) for the measurement of brain cell death. The vol-
219 ume of damaged area was calculated by using an image-analysis system
220 (NIH Image J). The damage regions were considered as completely
221 white areas. Brain swelling was calculated as the ratio of volumes be-
222 tween right and left hemisphere sections.

223 *2.11. Statistical analysis*

224 Statistical analysis was performed by one-way analysis of variance
225 (ANOVA) followed by Dunnett's multiple comparison tests. Data are
226 presented as mean ± S.D.

3. Results

3.1. PEGylated liposomes accumulated in ischemic region after reperfusion

To determine therapeutic time window for PEGylated liposomal 2
usage, we examined the time course of changes in the cerebral distri- 2
bution of fluorescence-labeled PEGylated liposomes with DiIC$_{18}$ after 2
reperfusion *ex vivo* (Fig. 1A–E). t-MCAO rats were injected with DiI- 2
labeled PEGylated liposomes via a tail vein at 0, 1, 3, 6 or 24 h after 2
the start of reperfusion; and then brain sections were prepared 2
from them at 1 h after the injection. The fluorescence of the labeled 2
liposomes in the brain sections was then observed with an *in vivo* im- 2
aging system. The accumulation of DiI-labeled PEGylated liposomes 237
in the ischemic hemisphere was detected in and around the striatum 238
immediately after the start of reperfusion. The most abundant locali- 239
zation of DiI-labeled PEGylated liposomes was observed when the 240
liposomes were injected at 3 h after reperfusion (Fig. 1C). A small 241
amount of liposomal fluorescence was observed when the liposomes 242
were injected at 6 h of reperfusion (Fig. 1D), but hardly any was 243
detectable when the injection was done at 24 h of reperfusion 244
(Fig. 1E). Therefore, it appears that the region where the PEGylated li- 245
posomes accumulated in the ischemic hemisphere gradually spread 246
as time passed until around 3 h after the start of reperfusion. Next, 247
t-MCAO rats were injected with DiI-labeled PEGylated liposomes im- 248
mediately after the start of reperfusion, and their brains were dissect- 249
ed at 24 h post injection to examine the cerebral distribution of the 250
liposomes. The results revealed that the fluorescence of DiI-labeled 251
PEGylated liposomes was detected in the ischemic hemisphere at 252
24 h (Fig. 1F). These data suggest that PEGylated liposomes were 253
retained in the ischemic hemisphere for an extended period of time. 254
Moreover, higher liposomal fluorescence intensity was observed in 255
the brain sections taken at 24 h after injection than in those taken 256
with at 1 h after injection (Fig. 1B, F), suggesting that the PEGylated 257
liposomes gradually accumulated in the brain parenchyma due to 258
the EPR effect. To quantify the accumulated amount of PEGylated 259
liposomes that accumulated in the ischemic hemisphere, we labeled 260
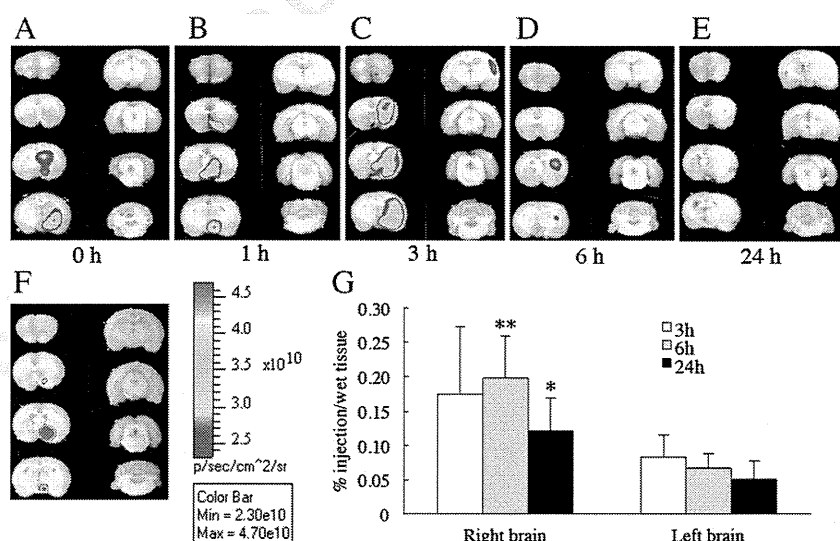
Fig. 1. Time course of PEGylated liposome localization in the brain of t-MCAO model rats. A–E) The t-MCAO rats were injected with DiI-labeled PEGylated liposomes (0.5 mL/rat *i.v.*)
at 0, 1, 3, 6 or 24 h of reperfusion; and then the rats were sacrificed at 1 h after the injection. F) The t-MCAO rats were injected with DiI-labeled PEGylated liposomes (0.5 mL/rat *i.v.*)
immediately after the start of reperfusion, and then their brains were dissected at 24 h after the injection. DiI-labeled PEGylated liposomes localized in the brain sections were ob-
served with IVIS. The left hemispheres of the brain slices are the non-ischemic side; and the right hemispheres are the ischemic side. Bar shows the relative levels of fluorescence
intensity, ranging from low (blue), to medium (green), to high (yellow, red). G) PEGylated liposomes were radiolabeled with [³H]cholesteryl hexadecyl ether. t-MCAO rats were
injected with [³H]-labeled PEGylated liposomes immediately after the start of reperfusion. These rats were sacrificed at 3, 6 or 24 h of reperfusion, their brains were removed,
and the radioactivity of the [³H]-labeled PEGylated liposomes in the ischemic and non-ischemic hemispheres of the brain was determined. The columns indicate the mean ± SD,
and the significant differences are as indicated: *p<0.05, ** p<0.01 vs. corresponding value for non-ischemic hemisphere. Fluorescence data represent of 5 independent animal
experiments, all of which demonstrated a similar profile of responses. (For interpretation of the references to color in this figure legend, the reader is referred to the web version
of this article.)

PEGylated liposomes with a radioisotope and injected them into t-MCAO model rats immediately after the start of reperfusion, and then sacrificed the animals at 3, 6 or 24 h after the injection (Fig. 1G and Fig. S1). The results revealed that PEGylated liposomes had a significantly higher degree of accumulation in the ischemic hemisphere compared with their amount in the non-ischemic hemisphere at 6 ($p = 0.007$) and 24 h ($p = 0.02$), but not at 3 h ($p = 0.08$), after the injection. Because cardiac perfusion was not performed in this experiment, both hemispheres included the radioactive PEGylated liposomes in the bloodstream. Thus, actual differences in the accumulation between ischemic and nonischemic hemispheres would be much larger. These data also indicate that PEGylated liposomes gradually accumulated in the ischemic region in a time-dependent manner. In addition, there was no significant difference between the hemispheres in the accumulation of PEGylated liposomes injected at 3 h after the start of reperfusion (Fig. S2). Taken together, these data suggest that the therapeutic time window for the use of PEGylated liposomes in the treatment of cerebral I/R injury is up to around 3 h after the start of reperfusion, with the most effective injection time point being regarded as immediately after the start of reperfusion.

3.2. AEPO-liposomes showed cytoprotective activity toward differentiated PC12 cells

After AEPO had been conjugated to DSPE-PEG-NHS, the conjugates were incubated with PEGylated liposomes; and then the AEPO-liposomes were purified by gel filtration (Fig. 2A). The optimal amount of AEPO to be used to modify the liposomes was determined by changing the molar ratio of AEPO to DSPE-PEG-NHS (Fig. S4A). Modification ratio of AEPO to PEGylated liposomes was approximately 35%, hence, 0.31 μg of AEPO was modified to 1 μmol of PEGylated liposomes. The average particle size and ζ-potential of AEPO-liposomes were 129 nm and 0.29 mV, respectively (Fig. S4B). The particle size of AEPO-liposomes showed little change in the presence of serum (Fig. S5). To examine the pharmacological activity of AEPO-liposomes, we evaluated the cytoprotective effects of them on PC12 cells by performing an MTT assay. PC12 cells are known to differentiate into nerve-like cells when treated with NGF; and the depletion of NGF induces programmed cell death (apoptosis) in the differentiated PC12 cells [28]. Depletion of NGF has also been observed in the striatum and cortex of the ischemic hemisphere of t-MCAO rats for 24 h of reperfusion [29,30]. In accordance with these findings, in the absence of NGF, the number of surviving differentiated PC12 cells was decreased to 32.1% of the number in the presence of the growth factor (Fig. 2B). Treatment of the cells with AEPO or AEPO-liposomes significantly suppressed the cell death observed in the absence of NGF (0.1 nmol/L AEPO vs. NGF (−) $p = 2.5 \times 10^{-5}$; 0.01, 0.1 or 1.0 nmol/L AEPO-liposomes vs. NGF (−) $p = 7.8 \times 10^{-5}$, 8.0×10^{-5}, 2.5×10^{-5}, respectively). The suppression of cell death brought about by the treatment with AEPO-liposomes occurred in a dose-dependent manner. The addition of PEG liposomes to PC12 cells in the absence of NGF did not significantly affect cell survival, indicating that the non-modified PEGylated liposomes were not neuroprotective.

3.3. Accumulation of AEPO in ischemic region was enhanced by liposomal DDS

AEPO was labeled with ^{125}I to trace the biodistribution of AEPO and AEPO-liposomes in the t-MCAO model rats. The rats were injected with ^{125}I-labeled AEPO or ^{125}I-labeled AEPO-liposomes via a tail vein immediately after the start of reperfusion, and then their distribution was evaluated at 3 and 24 h of reperfusion by measuring the accumulated radioactivity in each organ (Fig. 3A–D). The retention of AEPO in the bloodstream at 3 and 24 h after injection was significantly prolonged by liposomalization ($p = 0.002$ and $p = 0.04$, respectively; Fig. 3A–B). AEPO-liposomes showed higher accumulation in the liver than AEPO at both time points ($p = 2.1 \times 10^{-5}$ and $p = 0.008$ respectively). Fig. 3C and D show the amount of accumulation in the brain at 3 and 24 h after injection. The amount of AEPO-liposomes accumulated in the ischemic hemisphere was higher than that of AEPO at both times ($p = 2.4 \times 10^{-5}$ and $p = 0.004$, respectively). In addition, AEPO-liposomes accumulated significantly more in the ischemic hemisphere than in the non-ischemic hemisphere ($p = 0.007$ and $p = 0.04$, respectively); whereas AEPO showed no difference between the ischemic and non-ischemic hemispheres. Thus, liposomalization of AEPO prolonged the blood circulation time and increased the accumulation and retention of AEPO in the ischemic hemisphere at both 3 and 24 h of reperfusion.

3.4. AEPO-liposomes ameliorated neuronal apoptosis following cerebral I/R injury in rats subjected to t-MCAO

To evaluate the anti-apoptotic activity of AEPO-liposomes in the t-MCAO model rats, we observed TUNEL-positive cells in frozen brain
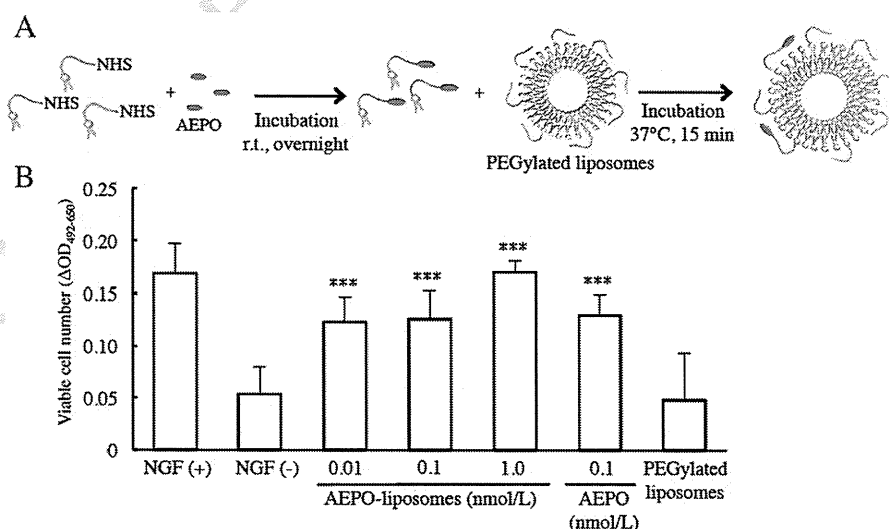

Fig. 2. Cytoprotective effect of AEPO-liposomes on PC12 cells. A) The strategy of modification of PEGylated liposomes with AEPO. B) PC12 cells were caused to differentiate by the addition of NGF at 100 ng/mL to culture medium supplemented with 0.5% HS. Following 5 days in culture for differentiation, the culture medium was changed to that without NGF, and each liposomal sample was added to the culture medium. After 5 additional days in culture, the number of viable cells was determined by performing the MTT assay. Data are presented as the mean ± S.D. ($n = 6$). Statistical differences were calculated by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests (comparison with NGF-free group). (***$p < 0.001$ vs. NGF [−]).
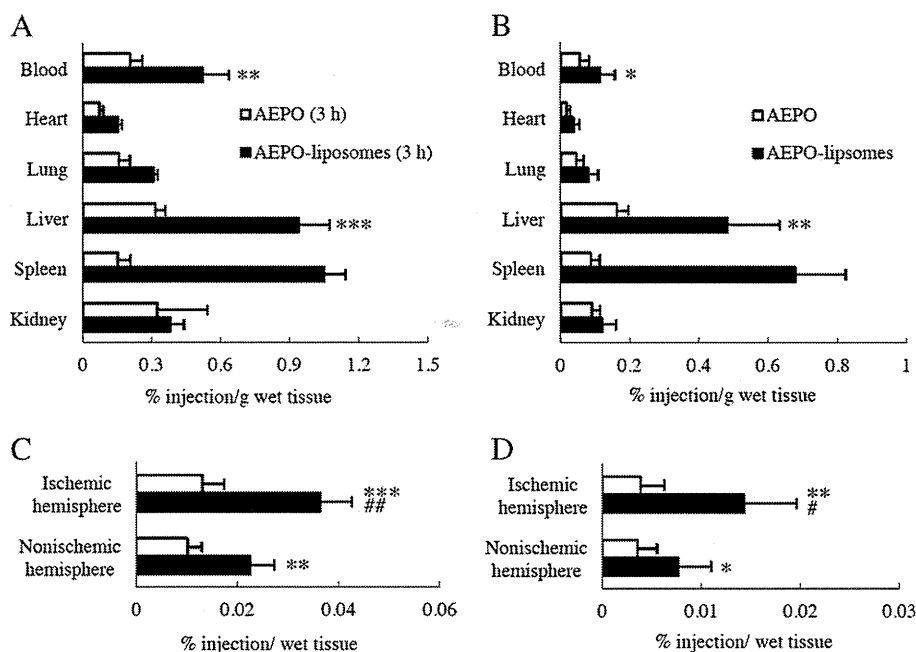
Fig. 3. Biodistribution of AEPO-liposomes in the t-MCAO model rats. AEPO was labeled with [125]I. The t-MCAO model rats were injected with ^{125}I-labeled AEPO or ^{125}I-labeled AEPO-liposomes via a tail vein. Biodistribution of each sample was determined by measuring the radioactivity in each organ at 3 (A and C) or 24 h (B and D) after the injection. Data are presented as the mean ± S.D. ($n = 5$). Significant differences are indicated as follows: * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. AEPO value, # $p<0.05$, ## $p<0.01$ vs. nonischemic hemisphere.

sections of striatum and cerebral cortex by confocal microscopy (Fig. 5A–D). In the non-ischemic hemisphere, almost no TUNEL-positive cells were observed in either the striatum or the cerebral cortex. However, in the ischemic hemisphere of the control (PBS-treated) group, many cerebral cells in both the striatum and the cerebral cortex were TUNEL positive. In contrast, in the AEPO-liposome-treated group, the number of TUNEL-positive cells in the striatum of the ischemic hemisphere was significantly reduced compared that of the control group ($p = 0.02$; Fig. 5A, C). On the other hand, no significant difference in TUNEL-positive cell number was found in the cerebral cortex ($p = 0.16$; Fig. 5B, D).

The therapeutic effect of AEPO-liposomes on the cerebral I/R injury in the t-MCAO model rats was examined by evaluating the volume of damaged brain, degree of brain swelling, and motor activity of rats at 24 h after the start of reperfusion (Figs. 4E–G, 5). As judged by TTC staining, AEPO-liposomes greatly reduced cerebral cell death compared with the control (PBS) and AEPO ($p = 2.3 \times 10^{-5}$ and $p = 0.003$, respectively; Fig. 5E, F). In particular, TTC-defined cerebral lesion in the striatum was strongly suppressed by the treatment with AEPO-liposomes. The volume of damaged brain was not changed by the PEGylated liposomes, indicating that PEGylated these liposomes were neither neuroprotective nor augmented the cerebral I/R injury. Brain edema, a life-threatening complication caused by cerebral I/R, was determined based on the difference between the volume of the right cerebral hemisphere and that of the left one. One hour of ischemia and 24 h of reperfusion increased ischemic hemisphere volume compared with the volume for the sham group (Fig. 5G). However, the brain swelling was significantly suppressed by the treatment with AEPO-liposomes ($p = 1.9 \times 10^{-5}$). Moreover, AEPO-liposomes clearly improved neurological function at 24 h of reperfusion ($p = 0.03$; Fig. 6). These results taken together indicate that AEPO-liposomes have the potential to improve stroke outcome and a patient's prognosis. It is also significant that these liposomes did not increase the hematocrit value (an indicator of blood viscosity), as such an increase is suggestive of a poor cerebral stroke outcome (Table S1). This indicates that liposomalization of AEPO did not stimulate hematopoiesis, despite affording an increase in the AEPO level in the blood circulation.

4. Discussion

We previously reported that obvious damaged area appeared at 3 h of reperfusion in our t-MCAO model rats [31]. In the present study, using the same model rats, we observed that PEGylated liposomes injected after the start of reperfusion accumulated in the brain parenchyma quickly thereafter. These results suggest that liposomal drug delivery to an ischemic region after reperfusion is possible before the occurrence of obvious brain damage. A number of neuroprotective drugs have failed in clinical trials due to inadequate setting of the therapeutic time window [32]. We speculated that the therapeutic time window of nanoparticles would be up to about 3 h after the start of reperfusion, since the accumulation of PEGylated liposomes in the ischemic hemisphere was low in the case of the injection given at 6 h of reperfusion.

Our results indicated that there was little accumulation of PEGylated liposomes in the ischemic region when injected more than 6 h after reperfusion had begun. One possible reason for this poor accumulation is the interruption of blood flow. It has been reported that I/R impedes the microcirculation [33]. Two hours of ischemia and 6 h of reperfusion have been shown to induce the contraction of pericytes by causing oxidative-nitrative stress. This phenomenon may limit drug delivery to an ischemic region. This finding suggests that pericyte contraction may interrupt the circulation of nanoparticles in the ischemic hemisphere. This hypothesis would thus explain why PEGylated liposomes given at 24 h after the start of reperfusion did not accumulate in the ischemic region. Elimination of this oxidative-nitrative stress might possibly prolong the therapeutic time window of liposomal agents.

Several reports have described the usefulness of AEPO for the treatment of cerebral stroke in rodent models. Wang et al. showed that the intraperitoneal injection of AEPO (80 µg/kg) before focal ischemia reduces infarct volume in their hypoxic-ischemic model rats by suppressing ERK activation and up-regulating SNAP-25.[25] Another report showed that AEPO (44 µg/kg) administered intravenously at the restoration of cerebral bloodstream in focal ischemia model rats decreases infarct volume measured at 24 h after the injection [24].