Manuscript title

Combined gene therapy of vascular endothelial growth factor and apelin for a chronic cerebral hypoperfusion model in rats

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Abstract

OBJECT: The aim of this study is to evaluate whether the combined gene therapy of vascular endothelial growth factor (VEGF) and apelin in indirect vasoreconstructive surgery enhances brain angiogenesis in a chronic cerebral hypoperfusion model in rats.

METHODS: A chronic cerebral hypoperfusion model induced by the permanent ligation of bilateral common carotid arteries (CCAs) in rats was employed in this study. Seven days after the ligation of bilateral CCAs, encephalo-myo-synangiosis (EMS) and plasmid administration in the temporal muscle were performed. Rats were divided into four groups by injected plasmids (i.e., LacZ group, VEGF group, apelin group, and VEGF/apelin group). Fourteen days after EMS, immunohistochemical analyses of cortical vessels were performed. Seven days after EMS, protein assays of cortex and attached muscle were performed.

RESULTS: In the VEGF group and the VEGF/apelin group, the total number of blood vessels in the cortex was significantly larger than that in the LacZ group ($p < 0.05$, respectively). In the VEGF/apelin group, larger vessels were induced than in the other groups ($p < 0.05$, respectively). Apelin protein was not detected in the cortex of any groups. In the attached muscle apelin protein was detected only in the apelin group and the VEGF/apelin group. Immunohistochemical analysis revealed that apelin and its

receptor APJ were expressed on the endothelial cells 7 days after the ligation of CCAs. CONCLUSIONS: The combined gene therapy of VEGF and apelin with EMS in a chronic cerebral hypoperfusion model in rats can enhance angiogenesis. This has potential as a feasible treatment option for moyamoya disease in a clinical setting.

Background and Purpose

Moyamoya disease (MMD) is a chronic, progressive cerebrovascular disease characterized by stenosis or occlusion of the bilateral supraclinoid internal cerebral arteries and the development of an abnormal vascular network called moyamoya vessels at the base of the brain that blocks cerebral flow.[21] Indirect bypass surgeries such as enchepalo-myo-synangiosis (EMS) are mostly performed for pediatric patients with MMD. The critical issue in indirect bypass for MMD is the fact that the amount of collateral circulation by indirect bypass surgery is sometimes insufficient for most adult and some pediatric cases.[20, 25, 27, 32, 39]

To develop sufficient collateral circulation, we have investigated the effect of EMS combined with vascular endothelial growth factor (VEGF) gene administration to the temporal muscles in a chronic ischemia model in rats.[15, 22] Adding the EMS surgery for bilateral common carotid arteries (CCAs) ligation, we have simulated the indirect bypass surgery for MMD. The data demonstrated that EMS with administration of plasmid human VEGF significantly increased angiogenesis in the cerebral cortex compared to EMS without administration of the VEGF gene.[15, 22] The over-expression of VEGF can, however, introduce a risk of immature vessel formation that result in plasma leakage and angioma formation.[4, 11, 24, 35]

Apelin has been identified as the endogenous ligand of the orphan G protein-coupled receptor APJ that is expressed in the cardiovascular and central nervous systems.[29] The apelin-APJ system is involved in a wide range of physiological activities, such as heart contractility and blood pressure regulation[6], appetite and drinking behavior,[23] neuroprotection,[30] and angiogenesis.[7, 14, 17, 18, 26] It has been reported that apelin together with VEGF effectively induced functional vessels that are larger than those with VEGF alone in the hind limb ischemia model.[18]

In this report, we evaluated whether the combined gene therapy of VEGF and apelin in indirect vasoreconstructive surgery enhances brain angiogenesis in a chronic cerebral hypoperfusion model in rats.

Materials and Methods

Animals and Surgical Procedures

All animal procedures in this study were specifically approved by the Institutional Animal Care and Use Committee of Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences (approval number: OKU-2013158).

Adult male Wistar rats (9-11 weeks old, weighing 250-350 g) were used for the experiments. Under general anesthesia with 2.0% halothane in a mixture of 40% oxygen

and 60% nitrous oxide gas the ~~common carotid arteries (~~CCAs~~)~~ were carefully separated from the sympathetic and vagal nerves using a ventrocervical incision. Bilateral CCAs were ligated with 3-0 silk sutures. The body temperature in the rats was maintained close to 37°C throughout the procedure and by using a heating pad. Sham operations involved skin incision and exteriorization of bilateral CCAs without CCA ligation. An interval of 7 days was allowed for postoperative recovery (Figure 1A).

Seven days after the bilateral CCAs ligation, the rats underwent EMS surgery (Figure 1B). The period between CCAs ligation and the EMS surgery is short, 7 days. This period was recruited because bilateral CCAs ligation reduces CBF to 35-50% of the control level, and CBF start to recover at 1 week. We thought that delayed EMS surgery after the beginning of CBF recover could be a negative effect for angiogenesis. In past literatures from other institutions, this period was recruited to develop similar model, too. Under the general anesthesia with the intraperitoneal administration of pentobarbital sodium (45mg/kg), the rats were placed in a stereotactic apparatus with the top of the skull positioned horizontally. After the midline linear incision, the right temporal muscle was detached from the temporal bone. Craniotomy was then performed in the temporo-parietal region using a dental drill. The dura mater was carefully opened and removed with no disruption of the brain surface (Figure 1C). The exposed brain

surface was covered with the muscle flap (Figure 1D). Plasmid injection in the temporal muscle was performed using GenomOne-Neo transfection reagent (Ishihara Sangyo) according to the manufacturer's protocol. Rats were divided into four groups by injected plasmids (i.e., LacZ group, VEGF group, apelin group, and VEGF/apelin group). Quantity of plasmid was 25μg in each group. In our previous report, we simply injected 50μg of VEGF plasmid into the temporal muscle, and found significant increase of capillary density.[22] Moreover, we performed optimal dose analysis that demonstrated the maximal angiogenic effect occurred with a 100μg dose of VEGF plasmid.[15] In comparison study of transfection efficacy between naked plasmid and method using GenomOne-Neo transfection reagent, transfection efficacy of this method was more than four times than injection of naked plasmid.[38]

Immunohistochemical Analysis

Bilateral CCAs ligation and sham rats were euthanized with an overdose of pentobarbital (100mg/kg) 1 week after surgery (Figure 1A), and EMS model rats were euthanized 2 weeks after EMS (Figure 1B). They were perfused transcardially with 200 ml of cold phosphate-buffered saline (PBS) and 100 ml of 4% paraformaldehyde (PFA) in PBS. The brain and transfected temporal muscle were removed and post-fixed in the

same fixative overnight at 4°C, and subsequently stored in 30% sucrose in PBS until completely submerged. Frozen coronal sections (17 μm thick) were cut from each specimen on a cryostat. The sections were thaw mounted on slides. Slides from the bilateral CCA ligation or sham operation without EMS surgery were evaluated with immunohistochemical analysis of endothelial cells (ECs) and apelin/APJ protein. Slides from the bilateral CCA ligation with EMS surgery 2 weeks after were evaluated with immunohistochemical analysis of ECs. The number per group was 8 in each group. Sections which include cortical surface were photographed at 10x magnification. The number of all vessels per field, percentage of vessel area per field and number of large vessels (>10 μm) per field were calculated in each photograph from the images using ImageJ.

For the immunohistochemical staining of ECs, after several rinses in PBS, slides were incubated in 10% fetal bovine serum in PBS for 1 h. Then, the slides were washed and incubated with an affinity-purified mouse monoclonal anti-endothelial cell antibody (RECA-1) with 1% fetal bovine serum for 2 h at room temperature. The slides were washed and incubated for 1 h with a Cy3 anti-mouse IgG antibody at 1:200 dilution at room temperature.

For the immunohistochemical staining of APJ and ECs, after several rinses in PBS,

slides were incubated in 10% fetal bovine serum in PBS for 1 h. Then, the slides were washed and incubated with RECA-1 and an affinity-purified rabbit polyclonal anti-APJ antibody with 1% fetal bovine serum for 2 h at room temperature. The slides were washed and incubated for 1 h with a Cy3 anti-mouse IgG antibody at 1:200 dilution and Alexa fulor anti-rabbit IgG antibody at 1:200 at room temperature.

For the immunohistochemical staining of apelin and ECs, after several rinses in PBS, slides were incubated in 10% normal goat serum in PBS for 1 h. Then, the slides were washed and incubated with an affinity-purified rabbit polyclonal anti-von Willebrand factor antibody and an affinity-purified mouse monoclonal anti-apelin antibody with 1% normal goat serum for 2 h at room temperature. The slides were washed and incubated for 1 h with an FITC anti-rabbit IgG antibody at a 1:300 dilution and a Cy3 anti-mouse IgG antibody at 1:300 dilution at room temperature.

Enzyme-linked Immunosorbent Assay (ELISA) Analyses

For protein assay, the CCAO and EMS models were quickly harvested after the decapitation of animals anesthetized with an overdose of pentobarbital (100 mg/kg, i.p.) 1 week after EMS surgery. The number per group was 2 in each group. Their brains and muscles were sliced with a thickness of 2mm. The brain tissue of the cortex was

punched out using a biopsy punch (3 mm hole, Kai Corporation and Kai Industries Co., Ltd., Japan). Brain and muscle tissues were then homogenized in T-PER (Pierce, Rockford, IL) and centrifuged at 10,000 G for 10 min at 4°C, and the supernatant was obtained. Induced VEGF and apelin levels of the brain and muscle of the CCAO and EMS models were measured using human VEGF ELISA and apelin-12 ELISA assay kits.

Statistical Analyses

The number of vessels and ELISA were evaluated statistically using single analysis of variance (ANOVA), with subsequent post hoc Tukey-Kramer ~~Fisher's protected least significance difference (PLSD)~~ test. Statistical significance was preset at $p < 0.05$.

Results

At the capillary level, the number of blood vessels in the VEGF and the VEGF/apelin groups was significantly higher than that in the LacZ group ($p < 0.05$, respectively) (Figure 2A, 2B). Percentage of vessel area per field in the VEGF/apelin group was significantly higher than that in the LacZ group ($p < 0.05$)(Figure 2C). Moreover, the number of large vessels in the VEGF/apelin group was significantly higher compared to

that in the LacZ, VEGF, and apelin groups ($p < 0.05$, respectively) (Figure 2D~~C~~).

The protein levels of VEGF and apelin in the attached muscle and the cortex 1 week after EMS were evaluated in all four groups. The protein levels of VEGF in the attached muscle in the VEGF and the VEGF/apelin groups tended to be higher than in the other groups but single ANOVA showed no significant difference ($p = 0.095$). VEGF protein in the cortex was not detected in any of the groups (Figure 3A). Apelin protein in the attached muscle was detected only in the apelin group and the VEGF/apelin groups. Apelin protein in the cortex was not detected in any of the groups (Figure 3B).

Immunohistochemical staining of the brain with monoclonal anti-apelin antibody and polyclonal anti-APJ antibody 7 days after occlusion of the bilateral CCAs revealed that ECs stained by RECA-1 or vWF antibody in the cortex express apelin or APJ (Figure 4).

Discussion

Indirect bypass surgery for moyamoya disease

Direct and/or indirect bypass surgery is often performed in patients with MMD as a surgical treatment. Although direct bypass surgeries such as superficial temporal artery-middle cerebral artery anastomosis are frequently performed in adult and

pediatric patients, direct bypass surgeries are sometimes difficult, especially in young children. Due to its easy and simple manner, indirect bypass surgery for MMD is a commonly used procedure to increase cerebral blood flow. Although it has been reported that indirect bypass surgeries are effective for pediatric and young adult patients with MMD, direct bypass surgery is the main treatment option for most adult patients with MMD.[27] The most important issue related to indirect bypass surgery for MMD is the fact that the amount of collateral circulation by surgery is sometimes insufficient for most adult and some pediatric cases.[20, 25, 27, 32, 39] An endogenous angiogenic factor may be involved in the development of collateral circulation. Park et al. demonstrated that the genotype of the VEGF allele was related to better collateral vessel formation after bypass surgery in patients with MMD.[33] These data indicate that the addition of an exogenous angiogenic factor to indirect bypass surgery could enhance the level of collateral vessels.

Gene therapy for a chronic cerebral hypoperfusion model

In this report, we confirmed that, in the VEGF group and the VEGF/apelin group, the total number of blood vessels in the cortex was significantly larger than that in the LacZ group. Moreover, in the VEGF/apelin group, larger vessels were induced than in the

other groups. In the hypoperfusion mode indirect bypass surgery without angiogenic factors could increase the number of vessels, and additional angiogenic factors could lead to a further increase.[1, 2, 12, 15, 19, 22, 31] Hechet et al. showed that EMS with VEGF expressing myoblasts for mice with unilateral ICA ligation improved not only the vessel density of the cortex but also the cerebrovascular reserve capacity.[12] Similar reports focused on vessel caliber size were, however, limited. Ohmori et al. reported that the encephalogaleosynangiosis (EGS) with granulocyte-colony stimulating factor (G-CSF) for hypoperfusion rats increased the number of smaller vessels.[31] We thought that the increase in the number of larger vessels was important factor in development of mature angiogenesis. Kidoya et al. reported that the apelin/APJ system spatially and temporally modulates caliber size enlargement during embryogenesis.[17] Some reports suggested that tissue hypoxia induced apelin expression on ECs.[7, 14, 17, 18] The mechanism of enlarged blood vessels by the apelin/APJ system was described as follows.[36] VEGF induces APJ expression on sprouted ECs, and angiopoietin-1 (Ang-1) induces apelin expression on ECs. Apelin induces the assembly of ECs and the proliferation of ECs with VEGF. Finally, APJ disappears on angiogenesis-inactive ECs and caliber size regulation finishes.

Newly formed vessels promoted by the over-expression of VEGF can be immature

and are at risk of tissue edema[11] or hemangioma.[35] Some papers reported combined gene therapy, such as VEGF and Ang-1, for ischemic hind limb models, myocardial infarction models, or acute cerebral ischemia models, and VEGF and apelin for ischemic hind limb models.[3, 5, 18, 34, 40, 41] The merits of the combined gene therapy of VEGF and apelin were reported to be the increase in vessel density and the maturation of newly developed vessels, including larger vessel formation and low permeability.[36] Some studies reported that VEGF-mediated permeability occurred through the disorganization of endothelial junction proteins, such as VE-cadherin.[10, 16] It was reported that apelin inhibited the down-modulation of VE-cadherin by VEGF, resulting in the suppression of hyperpermeability.[18] Although we reported the development of larger vessels, we could not confirm the suppression of hyperpermeability in the VEGF/apelin group. When we apply gene therapy to the indirect bypass of MMD, the development of mature newly formed vessels due to bypass surgery may lead to a reduction in adverse effects and an increase in cerebral blood flow.

Secreted proteins were detected only in muscles. This was also described in our previous report.[22] We thought that the injection of VEGF and apelin plasmids to the muscle increased angiogenesis not only in the muscle but also in the muscle-cortex interface. Then, the transpial vessel sprouted to the cortex. Similar models have reported

this mechanism.[12, 28] In particular, Nakamura et al. showed the mechanism of revascularization in an experimental model after the EMS of pigs.[28] During cerebral ischemia, the infiltration of inflammatory cells between the temporal muscle and the arachnoid membrane developed angiogenesis and led to revascularization between the external cerebral artery and the cerebral cortex artery.

Study limitations

This study has some limitations. First, the observational period of immunohistochemical analyses and protein assay were ~~was~~ short. Our previous studies and similar studies from other institutions reported results from evaluations of 2 to 4 weeks.[1, 12, 15, 19, 22, 31] In general, angiogenesis developed several months after indirect bypass surgery in patients with MMD. Future studies need to analyze collateral formation and protein assay in this model over a longer period.

Second, we could not conduct behavioral assessments in our model. Cognitive impairment can occur in patients with MMD.[9, 13] Assessment of the correlation between the development of collateral formation and the change in cognitive function in this model is desirable.

Third, we did not conduct measurement of vessel dysfunction, such as breakdown of

brain blood barrier, vessel leakiness, tight junction protein assessment, brain edema.

Fourth, we could not conduct blood flow measurements. In the past literatures, after bilateral CCAs ligation of rat, the greatest reduction in cerebral blood flow to 35-50% of the control level.[8, 37] In the future, we need blood flow measurement to show the effect of enhanced angiogenesis.

In the present study, we performed only vessel number analysis in short period, therefore, further analyses as described above are needed before the clinical application.

Conclusions

The combined gene therapy of VEGF and apelin with EMS in a chronic cerebral hypoperfusion model in rats can enhance mature angiogenesis. This could potentially be a feasible treatment option for MMD in a clinical setting.

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Disclosure

The authors report no conflicts of interest related to the materials or methods used in this study or the findings specified in this paper.

Figure legends

Figure 1. Time course of the experimental design and indirect bypass surgery

(A) Scheme showing the experimental design for the carotid artery occlusion model

(B) Scheme showing the experimental design for the indirect bypass surgery model

(C) Exposed brain after the craniotomy in the right temporo-parietal region

(D) Fascia attached (*) to the remaining parietal bone and brain covered with the temporal muscle (**)

CCAO: common carotid artery occlusion, EMS: encephalo-myo-synangiosis, IF: immuno-fluorescence

Figure 2. Analysis of angiogenesis of indirect bypass surgery model

(A) RECA-1 staining of the cortex in the four groups of the indirect bypass surgery model

(B) Quantitative evaluation of the number of vessels (*$p<0.05$)

(C) Percentage of vessel area per field (*$p<0.05$)

(D) Quantitative evaluation of the number of large vessels (more than 10 μm) (*$p<0.05$)

Figure 3. Results of ELISA analysis for human VEGF and apelin

(A) Human VEGF level in the cortex and attached muscle 1 week after indirect bypass surgery and administration of plasmids.

(B) Apelin level in the cortex and attached muscle 1 week after indirect bypass surgery and administration of plasmids

Figure 4. Immunohistochemical analysis of the cortex from the carotid artery occlusion model (CCAO) and sham-operated (sham)

(A) Immunohistochemical staining using anti-apelin antibody

(B) Immunohistochemical staining using anti-APJ antibody

CCAO: common carotid artery occlusion, vWF: anti-von Willebrand Factor antibody, RECA-1: anti-endothelial cell antibody

References

1. Anan M, Abe T, Matsuda T, Ishii K, Kamida T, Fujiki M, et al: Induced

angiogenesis under cerebral ischemia by cyclooxygenase 2 and hypoxia-inducible factor naked DNA in a rat indirect-bypass model. **Neurosci Lett** 409: 118-23, 2006

2. Anan M, Abe T, Shimotaka K, Kamida T, Kubo T, Fujiki M, et al: Induction of collateral circulation by hypoxia-inducible factor 1alpha decreased cerebral infarction in the rat. **Neurol Res** 31: 917-22, 2009

3. Arsic N, Zentilin L, Zacchigna S, Santoro D, Stanta G, Salvi A, et al: Induction of functional neovascularization by combined VEGF and angiopoietin-1 gene transfer using AAV vectors. **Mol Ther** 7: 450-9, 2003

4. Baumgartner I, Rauh G, Pieczek A, Wuensch D, Magner M, Kearney M, et al: Lower-extremity edema associated with gene transfer of naked DNA encoding vascular endothelial growth factor. **Ann Intern Med** 132: 880-4, 2000

5. Chae JK, Kim I, Lim ST, Chung MJ, Kim WH, Kim HG, et al: Coadministration of angiopoietin-1 and vascular endothelial growth factor enhances collateral vascularization. **Arterioscler Thromb Vasc Biol** 20: 2573-8, 2000

6. Dai T, Ramirez-Correa G, Gao WD: Apelin increases contractility in failing cardiac muscle. **Eur J Pharmacol** 553: 222-8, 2006

7. Eyries M, Siegfried G, Ciumas M, Montagne K, Agrapart M, Lebrin F, et al:

Hypoxia-induced apelin expression regulates endothelial cell proliferation and regenerative angiogenesis. **Circ Res** 103: 432-40, 2008

8. Farkas E, Luiten PG, Bari F: Permanent, bilateral common carotid artery occlusion in the rat: a model for chronic cerebral hypoperfusion-related neurodegenerative diseases. **Brain Res Rev** 54: 162-80, 2007

9. Festa JR, Schwarz LR, Pliskin N, Cullum CM, Lacritz L, Charbel FT, et al: Neurocognitive dysfunction in adult moyamoya disease. **J Neurol** 257: 806-15, 2010

10. Gavard J, Gutkind JS: VEGF controls endothelial-cell permeability by promoting the beta-arrestin-dependent endocytosis of VE-cadherin. **Nat Cell Biol** 8: 1223-34, 2006

11. Harrigan MR, Ennis SR, Masada T, Keep RF: Intraventricular infusion of vascular endothelial growth factor promotes cerebral angiogenesis with minimal brain edema. **Neurosurgery** 50: 589-98, 2002

12. Hecht N, Marushima A, Nieminen M, Kremenetskaia I, von Degenfeld G, Woitzik J, et al: Myoblast-mediated gene therapy improves functional collateralization in chronic cerebral hypoperfusion. **Stroke** 46: 203-11, 2015

13. Karzmark P, Zeifert PD, Tan S, Dorfman LJ, Bell-Stephens TE, Steinberg GK:

Effect of moyamoya disease on neuropsychological functioning in adults. **Neurosurgery** 62: 1048-51; discussion 1051-2, 2008

14. Kasai A, Shintani N, Oda M, Kakuda M, Hashimoto H, Matsuda T, et al: Apelin is a novel angiogenic factor in retinal endothelial cells. **Biochem Biophys Res Commun** 325: 395-400, 2004

15. Katsumata A, Sugiu K, Tokunaga K, Kusaka N, Watanabe K, Nishida A, et al: Optimal dose of plasmid vascular endothelial growth factor for enhancement of angiogenesis in the rat brain ischemia model. **Neurol Med Chir (Tokyo)** 50: 449-55, 2010

16. Kevil CG, Payne DK, Mire E, Alexander JS: Vascular permeability factor/vascular endothelial cell growth factor-mediated permeability occurs through disorganization of endothelial junctional proteins. **J Biol Chem** 273: 15099-103, 1998

17. Kidoya H, Ueno M, Yamada Y, Mochizuki N, Nakata M, Yano T, et al: Spatial and temporal role of the apelin/APJ system in the caliber size regulation of blood vessels during angiogenesis. **EMBO J** 27: 522-34, 2008

18. Kidoya H, Naito H, Takakura N: Apelin induces enlarged and nonleaky blood vessels for functional recovery from ischemia. **Blood** 115: 3166-74, 2010

19. Kim HS, Lee HJ, Yeu IS, Yi JS, Yang JH, Lee IW: The neovascularization effect of bone marrow stromal cells in temporal muscle after encephalomyosynangiosis in chronic cerebral ischemic rats. **J Korean Neurosurg Soc** 44: 249-55, 2008

20. Kim SK, Cho BK, Phi JH, Lee JY, Chae JH, Kim KJ, et al: Pediatric moyamoya disease: An analysis of 410 consecutive cases. **Ann Neurol** 68: 92-101, 2010

21. Kuroda S, Houkin K: Moyamoya disease: current concepts and future perspectives. **Lancet Neurol** 7: 1056-66, 2008

22. Kusaka N, Sugiu K, Tokunaga K, Katsumata A, Nishida A, Namba K, et al: Enhanced brain angiogenesis in chronic cerebral hypoperfusion after administration of plasmid human vascular endothelial growth factor in combination with indirect vasoreconstructive surgery. **J Neurosurg** 103: 882-90, 2005

23. Lee DK, Cheng R, Nguyen T, Fan T, Kariyawasam AP, Liu Y, et al: Characterization of apelin, the ligand for the APJ receptor. **J Neurochem** 74: 34-41, 2000

24. Lee RJ, Springer ML, Blanco-Bose WE, Shaw R, Ursell PC, Blau HM: VEGF gene delivery to myocardium: deleterious effects of unregulated expression.

Circulation 102: 898-901, 2000

25. Lee SB, Kim DS, Huh PW, Yoo DS, Lee TG, Cho KS: Long-term follow-up results in 142 adult patients with moyamoya disease according to management modality. **Acta Neurochir (Wien)** 154: 1179-87, 2012

26. Masri B, Morin N, Cornu M, Knibiehler B, Audigier Y: Apelin (65-77) activates p70 S6 kinase and is mitogenic for umbilical endothelial cells. **FASEB J** 18: 1909-11, 2004

27. Mizoi K, Kayama T, Yoshimoto T, Nagamine Y: Indirect revascularization for moyamoya disease: is there a beneficial effect for adult patients? **Surg Neurol** 45: 541-8; discussion 548-9, 1996

28. Nakamura M, Imai H, Konno K, Kubota C, Seki K, Puentes S, et al: Experimental investigation of encephalomyosynangiosis using gyrencephalic brain of the miniature pig: histopathological evaluation of dynamic reconstruction of vessels for functional anastomosis. Laboratory investigation. **J Neurosurg Pediatr** 3: 488-95, 2009

29. O'Carroll AM, Selby TL, Palkovits M, Lolait SJ: Distribution of mRNA encoding B78/apj, the rat homologue of the human APJ receptor, and its endogenous ligand apelin in brain and peripheral tissues. **Biochim Biophys Acta**

1492: 72-80, 2000

30. O'Donnell LA, Agrawal A, Sabnekar P, Dichter MA, Lynch DR, Kolson DL: Apelin, an endogenous neuronal peptide, protects hippocampal neurons against excitotoxic injury. **J Neurochem** 102: 1905-17, 2007

31. Ohmori Y, Morioka M, Kaku Y, Kawano T, Kuratsu J: Granulocyte colony-stimulating factor enhances the angiogenetic effect of indirect bypass surgery for chronic cerebral hypoperfusion in a rat model. **Neurosurgery** 68: 1372-9; discussion 1379, 2011

32. Pandey P, Steinberg GK: Outcome of repeat revascularization surgery for moyamoya disease after an unsuccessful indirect revascularization. Clinical article. **J Neurosurg** 115: 328-36, 2011

33. Park YS, Jeon YJ, Kim HS, Chae KY, Oh SH, Han IB, et al: The role of VEGF and KDR polymorphisms in moyamoya disease and collateral revascularization. **PLoS One** 7: e47158, 2012

34. Samuel SM, Akita Y, Paul D, Thirunavukkarasu M, Zhan L, Sudhakaran PR, et al: Coadministration of adenoviral vascular endothelial growth factor and angiopoietin-1 enhances vascularization and reduces ventricular remodeling in the infarcted myocardium of type 1 diabetic rats. **Diabetes** 59: 51-60, 2010

35. Schwarz ER, Speakman MT, Patterson M, Hale SS, Isner JM, Kedes LH, et al: Evaluation of the effects of intramyocardial injection of DNA expressing vascular endothelial growth factor (VEGF) in a myocardial infarction model in the rat--angiogenesis and angioma formation. **J Am Coll Cardiol** 35: 1323-30, 2000

36. Takakura N, Kidoya H: Maturation of blood vessels by haematopoietic stem cells and progenitor cells: involvement of apelin/APJ and angiopoietin/Tie2 interactions in vessel caliber size regulation. **Thromb Haemost** 101: 999-1005, 2009

37. Tanaka K, Ogawa N, Asanuma M, Kondo Y, Nomura M: Relationship between cholinergic dysfunction and discrimination learning disabilities in Wistar rats following chronic cerebral hypoperfusion. **Brain Res** 729: 55-65, 1996

38. Tashiro H, Aoki M, Isobe M, Hashiya N, Makino H, Kaneda Y, et al: Development of novel method of non-viral efficient gene transfer into neonatal cardiac myocytes. **J Mol Cell Cardiol** 39: 503-9, 2005

39. Touho H, Karasawa J, Ohnishi H, Yamada K, Shibamoto K: Surgical reconstruction of failed indirect anastomosis in childhood Moyamoya disease. **Neurosurgery** 32: 935-40; discussion 940, 1993

40. Toyama K, Honmou O, Harada K, Suzuki J, Houkin K, Hamada H, et al: Therapeutic benefits of angiogenetic gene-modified human mesenchymal stem cells after cerebral ischemia. **Exp Neurol** 216: 47-55, 2009

41. Yamauchi A, Ito Y, Morikawa M, Kobune M, Huang J, Sasaki K, et al: Pre-administration of angiopoietin-1 followed by VEGF induces functional and mature vascular formation in a rabbit ischemic model. **J Gene Med** 5: 994-1004, 2003