

## EPCs-Collagen Sponge Complex Promotes Neovascularization of Chronic Cerebral Ischemia following Multiple Burr Hole (MBH) Surgery

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### Abstract

**Objective:** The aim of this study was to explore the effect of endothelial progenitor cells (EPCs)-collagen sponge complex on the neovascularization of chronic cerebral ischemia following multiple burr hole (MBH) surgery.

**Methods:** Bone marrow-derived EPCs from 5 Fisher 344 (F344) inbred rats were cultured and amplified *in vitro*, then co-cultured to become the EPCs-collagen sponge complex. Chronic cerebral hypo-perfusion was induced in 30 inbred male F344 rats by permanent bilateral internal carotid artery occlusion (BICAO). The MBH operation was performed three days after BICAO surgery. Rats were then randomly divided into 3 groups (n=10 each group); EPCs-collagen sponge complex + MBH; collagen sponge + MBH and MBH group. Either EPCs-collagen sponge complex or collagen sponge was implanted at the burr hole site in the EPCs-collagen sponge complex + MBH and the collagen sponge + MBH group, respectively. Regional cerebral blood flow (rCBF) was measured by Laser Doppler Flowmetry (LDF), EPCs were tracked by fluorescein, and immune-histochemical analysis for the neovascularization was performed 21 days after MBH surgery.

**Results:** Before the MBH surgery, no differences of rCBF between the EPCs-collagen sponge complex + MBH group, the collagen sponge + MBH group and the MBH alone group (P>0.05) were found. The increase of rCBF in the EPCs-collagen sponge complex + MBH group (20.29 ± 10.21%) was significantly higher when compared with the other two groups 21 days after MBH surgery (7.39 ± 6.85% and 10.32 ± 6.27%, respectively; P<0.05). The amount of new blood vessels in the EPCs-collagen sponge complex + MBH group was also significantly greater than the other two groups (78.2 ± 4.7 vs. 48.7 ± 6.1 vs. 46.9 ± 7.3; P<0.05). The labeled EPCs were incorporated into the endothelial cells of vessels at the burr hole sites.

**Conclusion:** The EPCs-collagen sponge complex enhances angiogenesis after MBH surgery under chronic cerebral hypo-perfusion condition, and increases the blood flow perfusion in ischemic cerebral tissue.

**Keywords:** Angiogenesis; Endothelial progenitor cells; Collagen; Multiple burr hole (MBH) surge

### Introduction

Chronic cerebral ischemia often leads to cerebral infarction and requires bypass surgery. Multiple burr hole surgery (MBH), characterized as indirect bypass surgery, is relatively simple and safe and can create trans-galeal and/or transdural anastomoses from external arteries to an ischemic brain area via angiogenesis. This surgery has been demonstrated to be effective in the management of cerebral ischemia caused by moyamoya disease in children [1].

The degree of neo-anastomoses is a significant evaluation index for the effectiveness of the operation. However, suboptimal collateral vessel formation has been found in some adult moyamoya patients, as well as the unfavorable effect of stenosis or occlusion of cerebral arteries caused by atherosclerosis [1-3].

Therefore, successful promotion of postoperative neovascularization requires urgent attention. Several studies [4-6] have reported that endothelial progenitor cells (EPCs) promote the neovascularization of collateral vessels at the ischemic areas, thus further improving tissue perfusion. Collagen sponge is widely known as an excellent stereo-carrier for cell growth [7-9]. Whether EPCs-collagen sponge complex enhances angiogenesis following MBH surgery under chronic cerebral hypo-perfusion condition was investigated in this study, and whether this complex can further increase ischemic cerebral perfusion was also explored.

### Materials and Methods

#### Animals and grouping

Bone marrow from 5 Fisher 344 (F344) male inbred rats (250-300 g) was cultured and amplified *in vitro*. The remaining 30 F344 male inbred rats (250-300 g) were randomly divided into three groups (10 in

each group): EPCs-collagen sponge complex + MBH group, collagen sponge + MBH group and MBH alone group. All rats were anesthetized with 10% chloral hydrate via abdominal cavity injection, and were kept warm at the temperature of 37°C with heating pad when performing MBH surgery or bilateral internal carotid arteries ligation operation. Rats were provided by the Laboratory Animal Center affiliated to Shanghai Second Military Medical University. All animal protocols were approved by the Shanghai Second Military Medical University Animal Care Committee.

## Construction of the EPCs-collagen sponge complex

### Isolation and *in vitro* culture of EPCs

Animals were sacrificed through cervical dislocation after induction of general anesthesia with 10% chloral hydrate via abdominal cavity injection. Bone marrow cells were collected from the rat bone marrow by flushing the marrow cavity of bilateral femora with phosphate buffered saline (PBS). Mononuclear cells were separated by density gradient centrifugation (2000 r/min, 37°C) using lymphocyte isolation, centrifuged with PBS twice, added into EGM-2 MV medium (Lonza, USA), and plated on to fibronectin coated 25 mm<sup>2</sup> culture flasks. Thereafter, the cells were incubated (37°C, 5% CO<sub>2</sub>). 3 days later, non-adherent cells were removed along with the PBS, and fresh EGM-2 MV medium was added to the adherent cells. The morphology analysis of cultured cells was evaluated through phase contrast microscopy.

### Cellular staining

Fluorescent detection of the adherent EPCs was performed at day 7 since culture. Direct fluorescent staining was conducted to detect dual binding of TITC-labeled Ulex europaeus agglutinin-1 (TITC-UEA-1; Sigma) and 1,1'-dioctadecyl-3,3,3,3'-tetramethylindocarbocyanine (DiI)-labeled acetylated low density lipoprotein (DiI-ac-LDL; Invitrogen). Cells were first incubated with DiI-ac-LDL (12 ug/mL) for 4 h at 37°C and then fixed with 1% paraformaldehyde for 10 min. After washing 3 times with PBS, cells were incubated with TITC-UEA-1 (10 ug/mL) for 1 h. The samples were observed with an inverted fluorescent microscope (Leica, Wetzlar, Germany). Cells stained with red, green and double-positive fluorescence were identified as DiI-ac-LDL positive cells, TITC-UEA-1 positive cells and differentiating EPCs, respectively.

### Construction of EPCs-collagen sponge complex

Before the construction of the EPCs-collagen sponge complex, dual binding of CM-DiI and Hoechst 33342 was performed for the study of EPC tracking. Prior to cellular transplantation, cells were first washed with PBS 3 times and incubated with Hoechst 33342 (10 ug/mL) in PBS for 15 min, and then incubated with CM-DiI (2.5 ug/mL) in PBS at 37°C for 5 min and 4°C for 15 min. All samples were observed with an inverted fluorescent microscope (Leica, Germany). Cell membrane stained with red fluorescence and blue fluorescence was identified as CM-DiI positive cells and Hoechst 33342 positive cells, respectively.

Cells were then detached from the culture flask using Trypsin, washed with PBS and centrifuged. Following cell counting by hemocytometer, 1×10<sup>6</sup> cells were placed on top of a sterile gelfoam sponge (5 mm × 5 mm). The media was supplemented with EGM-2 MV (Lonza, USA), and cells were sub-cultured in 6-well plates at 37°C under 5% CO<sub>2</sub> atmospheric conditions.

## Chronic cerebral hypo-perfusion model

Bilateral internal carotid arteries (ICAs) were exposed via a midline cervical incision, sparing the sympathetic and vagal nerves. Ligation of bilateral ICAs was performed with 4-0 silk sutures.

### MBH surgery and implantation of the EPCs-collagen sponge complex or collagen sponge

At day 3 after the permanent bilateral internal carotid artery occlusion (BICAO), all rats were fixed in prone position with a stereotactic head holder. The scalp was then incised at the parieto-occipital midline, the galea and periosteum peeled off the skull, and the galeal flap prepared. A 6 mm<sup>2</sup> craniectomy was performed by drilling just behind the coronal suture, 1 mm lateral from the midline, and dural incision was performed with microsurgical forceps and a microscope for protection of brain tissue. EPCs-collagen sponge complex or collagen sponge was then placed into bone defects.

### Measurement of rCBF

The rCBF was measured 4 times: before, immediately after BICAO, before encephalo galeo syngangiosis (EGS) and 21 days after EGS. The rCBF was measured with a transcranial laser Doppler flowmeter (LDF) (Moor, UK). Rats were anesthetized with halothane and fixed in a stereotactic head holder. A midline scalp skin incision was made, and the flowmeter probe was fixed. rCBF was measured and calculated in each rat at the bilateral frontal lobe and the cerebellum. The calculated value percentage of each frontal rCBF was compared with the ipsilateral rCBF of the cerebellum.

### Histology and immunohistochemistry

Rats were deeply anesthetized 21 days after BICAO, and their aortas perfused with 4% paraformaldehyde in PBS. Brain tissue (along with EGS tissue) was removed, fixed with 4% paraformaldehyde for 6 h, dehydrated with sucrose and embedded in optimum cutting temperature compound. Coronal sections measuring 5 mm and including EGS tissue were obtained from each brain.

The sections were sequentially incubated with antibody against von Willebrand factor (vWF; 1: 200), and stained with hematoxylin and eosin (H&E) for histological evaluation. Sections were photographed at 40x magnification. Five photographs from different sites in the cerebral cortex were obtained from each brain section (n = 10). The amount and diameter of vWF-positive vessels in each section were recorded. Sections were analyzed blindly by 2 independent co-authors.

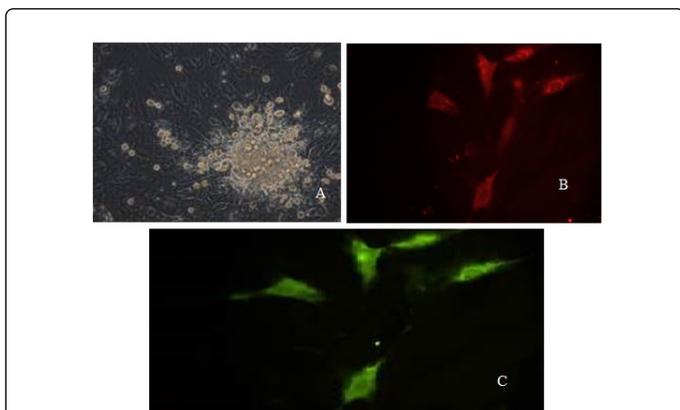
### Data analysis

Data are expressed as mean ± standard error (SE). Alterations in laser Doppler flowmetry (LDF) were calculated as percentage of measured blood flow/ipsilateral cerebellar blood flow. Student's t-test was performed and statistical significance was considered as less than 0.05.

## Results

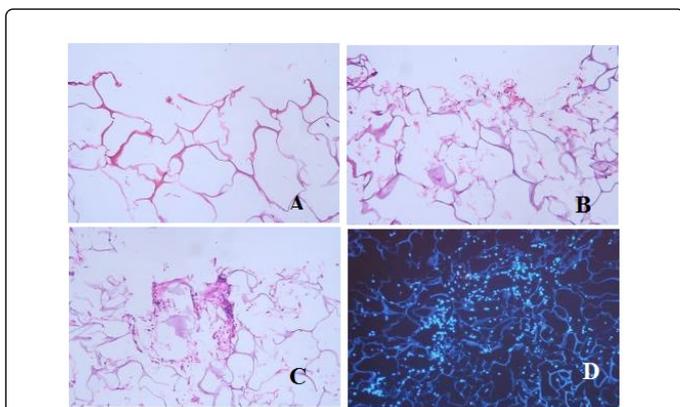
Characteristics of EPCs cultured *in vitro* and the construction of EPCs-collagen sponge complex After 48 hours *in vitro* culture on fibronectin coated flasks, adherent cells showed colony-like growth (Figure 1A). At day 7 of culture, the cells covered approximately 80% of

the flask area. The cultured cells had the function of phagocytizing acLDL and binding UEA-1, the same with endothelial cells.



**Figure 1:** Adherent cells showed colony-like growth (A). Endocytosis of acLDL (red fluorescence) (B) and binding to UEA (green fluorescence) (C) identified EPCs. All images are shown at 40x magnification.

Plating  $1 \times 10^6$  EPCs on to the collagen sponge in an *in vitro* co-culture model. At day 7, small amount of cells attached to the collagen sponge. However, at day 14 greater amounts of cells attached to the collagen sponge, grew and formed the complex.

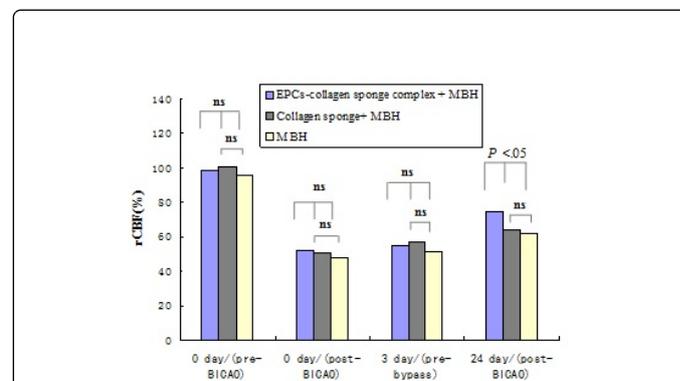


**Figure 2:** Collagen sponge has a good biocompatibility with EPCs, which is a successful *in vitro* three dimensional scaffold for EPCs culture. H&E staining of blank collagen sponge (A). H&E staining of EPCs-collagen sponge complex at day 7, few cells attached to the collagen sponge and amplified (B). H&E staining at day 14, many cells grew and attached to the collagen sponge (C). Nucleus stained blue was identified as Hoechst 33342 labeled positive cells at day 14 (D). All images are shown at 40x magnification.

### Changes of rCBF

No significance of the changes of rCBF between three groups before and after BICAO and pre-MBH surgery ( $P > 0.05$ ) was observed. 21 days after MBH surgery, the rCBF% of the EPCs-collagen sponge complex + MBH group was significantly higher ( $74.80 \pm 7.85\%$ ) than that of the other two groups (collagen sponge + MBH group,  $64.07 \pm$

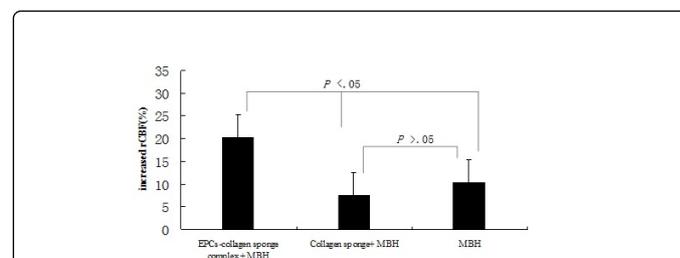
$7.05\%$  and MBH alone group,  $61.63 \pm 10.12\%$ ,  $P < 0.05$ ) (Figure 2 and 3). The increase in the rCBF% of the EPCs-collagen sponge complex + MBH group ( $20.29 \pm 10.21\%$ ) was higher than that of the other two groups (collagen sponge + MBH group,  $7.39 \pm 6.85\%$  and MBH alone group,  $10.32 \pm 6.27\%$ ,  $P < 0.05$ ) (Figure 4 and 5).



**Figure 3:** The rCBF in three groups before and after BICAO, pre- and post-MBH. The rCBF% of the EPCs-collagen sponge complex + MBH group was significantly higher than the other two groups 21 days after MBH ( $P < 0.05$ ). Data are presented as mean  $\pm$  SE. ns: not significant.

### Histological assessment of fluorescence-labeled EPCs

Both Hoechst 33342 and CM-DiI fluorescence labeling was used to identify the EPCs of the collagen sponge complex to confirm that the EPCs had reconstructed new vessels at the burr hole sites. Dual-fluorescently labeled EPCs were detected from tissue located at areas between the brain and galea at day 21 after MBH surgery (Figure 6).

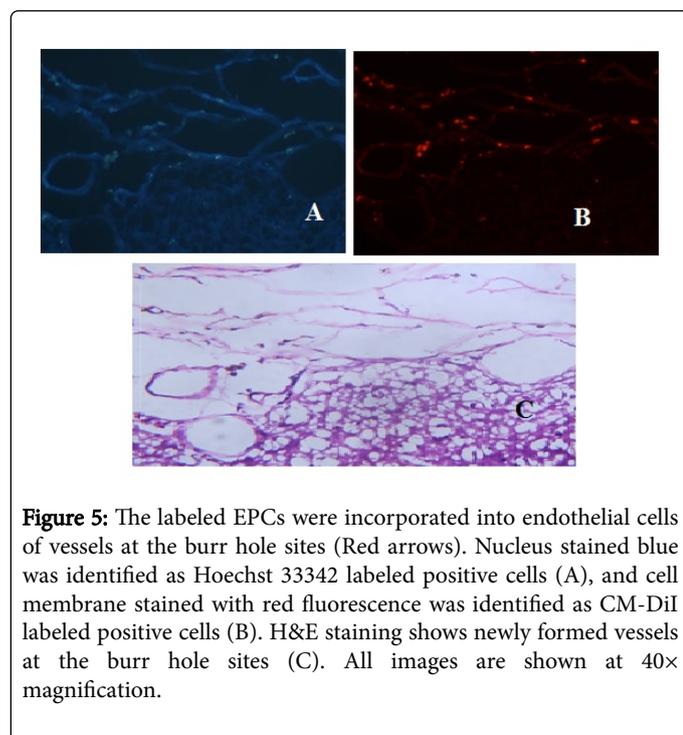


**Figure 4:** The rCBF% 21 days after MBH surgery was increased in each group. The increase in rCBF% in the EPCs-collagen sponge complex + MBH group was significantly higher than the other two groups. Data are presented as mean  $\pm$  SE.

### Immunohistochemistry

Immunohistochemistry staining for von Willebrand factor (vWF) protein expression, a marker of angiogenesis, was performed in each of the experimental groups at day 21 after MBH surgery. A boundary zone between the cerebrum and the galea tissue was identified. vWF protein positive vessels staining was observed visually with a microscope under 40 x magnification. The EPCs-collagen sponge complex + MBH group had significantly more new vessels

(angiographic score:  $78.2 \pm 4.7$  vs.  $48.7 \pm 6.1$  vs.  $46.9 \pm 7.3$ ,  $P < 0.05$ ) and more intense positive staining for vWF protein.



**Figure 5:** The labeled EPCs were incorporated into endothelial cells of vessels at the burr hole sites (Red arrows). Nucleus stained blue was identified as Hoechst 33342 labeled positive cells (A), and cell membrane stained with red fluorescence was identified as CM-Dil labeled positive cells (B). H&E staining shows newly formed vessels at the burr hole sites (C). All images are shown at 40 $\times$  magnification.

## Discussion

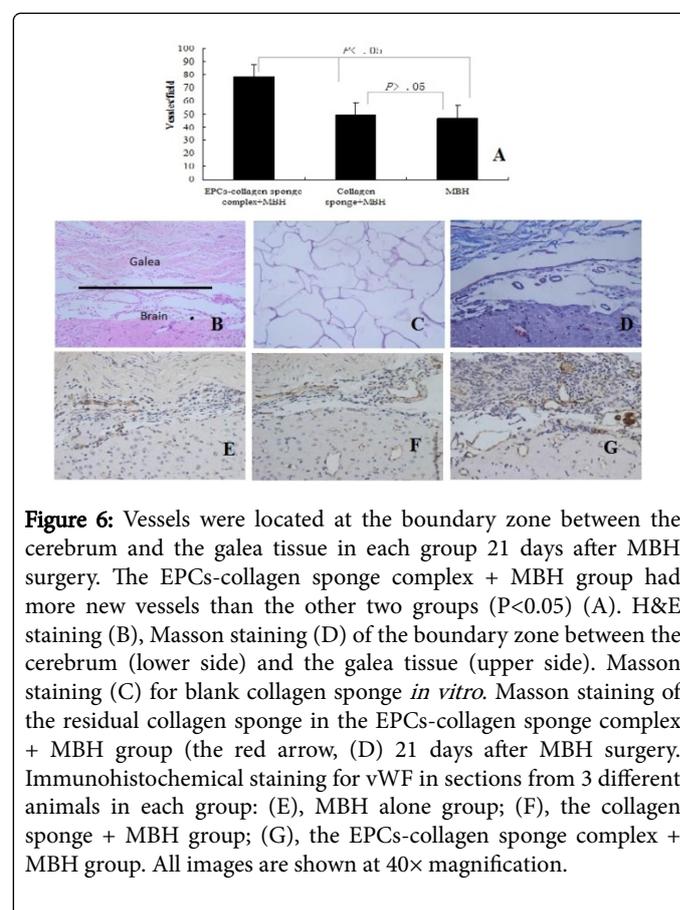
The rat BICAO model utilized in this study effectively simulated human chronic cerebral hypo-perfusion conditions of moyamoya and atherosclerosis as previously reported [10,11]. In this study, MBH surgery and indirect revascularization were performed on the 3rd day after BICAO to better simulate human cerebral hypo-perfusion disease. Furthermore, preservation of the blood flow of the external carotid artery was beneficial in fundamental research of the neovascularization of chronic cerebral ischemia following MBH surgery. Ohta et al. [10] showed that this model simulates human cerebral hypo-perfusion disease better at day 3 after BICAO.

Interpretation of this study is as follows: Firstly, EPCs-collagen sponge-complex and collagen sponge are biocompatible, as indicated in this study. A satisfactory complex by co-culture *in vitro* was also obtained. Secondly, EPCs, the precursor cells of neo-endothelial cells, promote the neovascularization of the collateral vessels at ischemic brain areas. And finally, EPCs-collagen sponge complex promote neo-anastomoses from external arteries to an ischemic brain via angiogenesis under hypo-perfusion condition after MBH surgery, then further increase the ischemic cerebral perfusion.

The *in vitro* spindle-shaped cells, commonly known as EPCs, were first discovered and named by Asahara et al. [12]. In this study, the EPC cells expressed endothelial cell antigen and formed cord-like structures. EPCs transplantation, as previous studies reported, successfully improve capillary density and promote neovascularization of ischemic tissues [5,13,14]. The effects of EPCs-collagen sponge complex in the neovascularization of the chronic cerebral ischemia remain to be reported.

The results of the current study indicate that the EPCs-collagen sponge complex promotes neovascularization of the collateral vessels from the external carotid system to ischemic brain tissue following MBH surgery. As precursor cells, EPCs play an important role in neo-angiogenesis and vasculogenesis [12], particularly in the neovascularization and remodeling of collateral vessels [15,16].

Unsatisfactory quality and quantity of EPCs in moyamoya or atherosclerosis [17], diabetes and incremental age [18] are key factors influencing the efficiency of neovascularization. *In vitro* transplantation of EPCs has proved to be helpful in solving the problem of EPC deficiency, providing a sufficient reserve of EP cells for neovascularization. Previous studies have reported that collagen sponges are satisfactory three-dimensional fibrin matrices [9,19,20]. Bilic et al. [19] used 3D collagen I matrices and embedded preterm amnion epithelial to create a cell-matrix system that mimicked the structure of native amnion, which could also be used in the basic research of bone and skin tissue engineering [9,20].



**Figure 6:** Vessels were located at the boundary zone between the cerebrum and the galea tissue in each group 21 days after MBH surgery. The EPCs-collagen sponge complex + MBH group had more new vessels than the other two groups ( $P < 0.05$ ) (A). H&E staining (B), Masson staining (D) of the boundary zone between the cerebrum (lower side) and the galea tissue (upper side). Masson staining (C) for blank collagen sponge *in vitro*. Masson staining of the residual collagen sponge in the EPCs-collagen sponge complex + MBH group (the red arrow, (D) 21 days after MBH surgery. Immunohistochemical staining for vWF in sections from 3 different animals in each group: (E), MBH alone group; (F), the collagen sponge + MBH group; (G), the EPCs-collagen sponge complex + MBH group. All images are shown at 40 $\times$  magnification.

Collagen sponges containing the Arg-Gly-Asp (RGD) sequence induce cell adhesion, cellular proliferation and differentiation, thus promoting neo-angiogenesis [21,22]. The advantages of this collagen sponge complex as scaffolds in MBH surgery include: 1) collagen sponges induce cell adhesion and cell proliferation inside pores, and prevent cell loss as seen by the increased flow of CSF; 2) this collagen sponge complex performed as a bridge grafting between dural lining and brain tissue as collagen sponges placed into the dural incision significantly increased EPCs proliferation, thereby stimulating angiogenesis; 3) the complex transplantation at bone defects positions EPCs at ischemic areas, thus avoiding bone marrow mobilization and

homing, and increases initial seeding density and 4) the results of the study show that the *in vitro* co-culture model has excellent biocompatibility, and that at day 14 this *in vitro* modification produces a capillary vascular bed. Consequently, this study shows that for tissue engineering purposes, the *in vitro* co-culture model, which combines EPCs and collagen sponges along with the transplantation of EPCs and capillary vascular beds in bone defects, promotes neo-angiogenesis, otherwise known as the cellular-level vascular bypass.

Antioxidant, a molecule that inhibits the oxidation of other molecules, has been reported for therapeutic use in several disorders [1]. However, clinical trials of pharmacological neuro-protective effect in stroke were disappointing. Till now, two endogenous mechanisms have been identified, namely ischemic pre-conditioning and ischemic post-conditioning. The neuro-protective concept of preconditioning is based on the fact that a transient, non-injurious episode of ischemia will protect the brain from a subsequent longer ischemic shock. Post-conditioning, a modified reperfusion subsequent to a prolonged ischemic episode, may also confer ischemic neuro-protection [23].

It is commonly accepted that MBH surgery and indirect revascularization is a simple, safe and generally non-invasive procedure of brain, with no risk of hyper-perfusion syndrome and limitation of vessel diameter and location, and is suitable for pediatric patients suffering from Moyamoya disease [1,24]. Previous studies have reported that, MBH surgery is an effective procedure in childhood cases of Moyamoya disease, which will decrease the recurrence rate of ischemic events following surgery [1,25].

However, the therapeutic effect is unsatisfactory for cerebral hypoperfusion resulting from major cerebral vessel stenosis or occlusion in adult Moyamoya disease or atherosclerosis [1,3]. Indeed, several studies have speculated that EPCs deficiency and/or dysfunction is associated with inactive angiogenesis [12]. In coordination with those results, EPCs-collagen sponge complex + MBH therapy might promote neovascularization following MBH surgery in adult Moyamoya or atherosclerosis-relevant cerebral hypo-perfusion.

## Conclusion

EPCs-collagen sponge complex enhances neo-angiogenesis at the burr hole site in a rat model of cerebral hypo-perfusion. Furthermore, the EPCs-collagen sponge complex increases blood flow perfusion in rat ischemic cerebral tissue.

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