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·Original Article·

# Small-animal PET demonstrates brain metabolic change after using bevacizumab in a rat model of cerebral ischemic injury

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## ABSTRACT

To evaluate the effect of bevacizumab on cerebral ischemia, we used 2-deoxy-2-$^{18}$F-fluoro-*D*-glucose ($^{18}$F-FDG) small-animal positron emission tomography (PET) in the middle cerebral artery occlusion (MCAO) rat model. After baseline neurologic function tests and PET studies, MCAO Sprague-Dawley rats received bevacizumab or normal saline (controls). Weekly PET imaging and neurologic function tests showed that the $^{18}$F-FDG accumulation in the bevacizumab group was similar to that in the controls during the first 2 weeks, but lower than in controls at weeks 3 and 4. However, no difference was found in neurological scores between the groups. The number of von Willebrand factor-positive cells in the bevacizumab group was lower than that in controls. The expression of vascular endothelial growth factor was higher than in controls at week 4. These results suggested that bevacizumab does not influence functional recovery in this model of cerebral ischemia during a 4-week period, but inhibits vascular formation and metabolic recovery, which may be considered in cancer patients with a recent ischemic stroke.

**Keywords:** cerebral ischemia; bevacizumab; positron emission tomography; cancer

## INTRODUCTION

Cerebrovascular disease (CVD) and cancer are the top two causes of morbidity and mortality in aging populations[1-3]. Studies have shown that CVD is typically associated with ischemic stroke[4]. Ischemic stroke featuring functional disturbance and morphological damage of brain is caused by CVD, which affects the brain blood supply and leads to a cascade of metabolic alterations. Clinical presentation of CVD in cancer patients is common: 14.6% of such patients have pathologic evidence of CVD, and 7.4% show clinical symptoms[5]. Although current therapeutic strategies improve the survival rate and extend the lifetime of cancer patients, increased risk of ischemic stroke is frequently observed in the same individuals[6]. Therefore, exploring the therapeutic strategies for recent ischemic stroke in cancer patients is a serious challenge.

Currently, anti-angiogenic therapy is widely used in the treatment of various solid cancers. Increasingly, angiogenesis-targeting therapies have been developed by manipulating the vascular endothelial growth factor (VEGF) signaling pathway. Bevacizumab (Avastin, Genentech/Roche, Basel, Switzerland), a recombinant humanized monoclonal antibody against VEGF-A, is the first anti-angiogenic drug approved by the Food and Drug Administration (USA)[7] for metastatic colorectal cancer[8],

non-squamous non-small-cell lung cancer[8], glioblastoma[9], and metastatic renal cell carcinoma[10]. Many studies have demonstrated that bevacizumab therapy is associated with an increased risk of gastrointestinal perforation, wound healing complications, hemorrhage, arterial thromboembolism, and reversible posterior leukoencephalopathy syndrome[11-14]. Therefore, patients with a history of bleeding, cerebrovascular accident, thrombotic disorders, and gastrointestinal perforation were excluded from participation in clinical trials[11].

Interestingly, our recent clinical observations have shown that significantly increased numbers of elderly cancer patients treated with bevacizumab have a recent ischemic stroke. One study reported that prolonged bevacizumab treatment increases the risk of ischemic stroke[12]. However, its safety in cancer patients with recent ischemic stroke is still unknown. In the current study, we evaluated the effect of bevacizumab on recent ischemic stroke using a molecular imaging approach.

Positron emission tomography (PET) provides *in vivo* metabolic information based on imaging the distribution of positron-emitting radiopharmaceuticals[15-16]. PET can not only locate the area of infarction like MRI scanning, but also provide metabolic information, which has led to significant insights into various neurologic disorders, including dementias[17], movement disorders[18], epilepsy[19-20], brain tumors[21], neurologic infectious, and inflammatory diseases[22-23]. Furthermore, PET imaging in neurologic disease can detect pathologic changes preceding those seen with structural imaging techniques and even clinical symptoms[24]. 2-deoxy-2-$^{18}$F-fluoro-*D*-glucose ($^{18}$F-FDG), the most extensively used PET imaging tracer, can detect subtle changes of glucose metabolism after stroke[26]. In a previous study, we successfully used $^{18}$F-FDG small-animal PET to evaluate the metabolic recovery of the cerebral infarction in an ischemic stroke model[27]. In this study, we used $^{18}$F-FDG PET to assess the metabolic changes, along with related immunohistochemical and functional changes, in a rat model of cerebral ischemia treated with bevacizumab.

## MATERIALS AND METHODS

### Animal and Experimental Design

Eight adult male Sprague-Dawley rats weighing 240–270 g were randomly assigned into two equal groups: bevacizumab treatment and control groups. All animals underwent reperfusion at 90 min after middle cerebral artery occlusion (MCAO). Rats in the treatment group were administered bevacizumab (Avastin, Genentech/Roche Inc., South San Francisco, CA) *via* tail vein at 5 mg/kg on the day after MCAO. Similarly, all rats in the control group were administered normal saline (NS) in the same way. All rats underwent neurologic function testing followed by $^{18}$F-FDG PET scanning on day 1 (before injection) and weeks 1, 2, 3, and 4 (after injection). Immunohistochemical staining was performed immediately after the last $^{18}$F-FDG PET scan. All experiments were performed with the approval of the Institutional Animal Care and Use Committee of Zhejiang University School of Medicine.

### Middle Cerebral Artery Occlusion Procedure

MCAO was induced as previously described[27]. Briefly, animals were anesthetized intraperitoneally with 1.5% pentobarbital sodium (50 mg/kg). Body temperature was maintained at 37 ± 0.5 °C with a warm pad during the procedure. The right common, internal, and external carotid arteries were exposed. A 3-0 monofilament nylon suture with a rounded tip was inserted from the right common carotid into the internal carotid and then advanced 18-20 mm intracranially from the common carotid bifurcation in order to block the origin of the middle cerebral artery. Approximately 90 min after MCAO, reperfusion was allowed by withdrawal of the suture. Then the muscle and skin were sutured with 4-0 nylon. Animals were given buprenorphine (0.05 mg/kg, subcutaneously) every 8 h[28] for pain palliation during the first 24 h after operation.

### Evaluation of Neurological Deficits

Animals were subjected to a weekly behavioral test for 4 weeks, using the Garcia neurological grading method[29]. This evaluation is a composite of spontaneous activity (abnormal movement), climbing, forepaw stretching, symmetry in the movement of four limbs, proprioception, and response to vibrissae touch. The score is the sum of the six individual test scores and ranges from 3 to 18. A score of 3 represents the most severe behavioral deficits and 18 means normal behavior. Rats with scores ranging from 7 to 12 were used. Investigators were blinded to the animal group to avoid the bias effect.

### PET Scanning and Image Analysis

Rats were anesthetized with isoflurane (2%) and administered ~18.5 MBq (500 mCi) of $^{18}$F-FDG *via* tail vein just before bevacizumab or NS injection and at weeks 1, 2, 3, and 4 later. PET images were acquired with a microPET R4 scanner (Siemens Medical Solutions, Munich, Germany) for 10 min static acquisition at 40 min after $^{18}$F-FDG injection. Anesthesia was maintained during data acquisition. The images were reconstructed with a modified back-projection algorithm. $^{18}$F-FDG uptake was calculated as the percentage of injected dose per gram of tissue, using the AMIDE software package (version 9.2; Stanford University, Santa Clara County, CA). To assess the metabolic changes induced by MCAO, regions of interest (ROIs) 2 mm in diameter were identified in coronal images. The lesion-to-homologous contralateral normal region (L/N) ratios were calculated according to the following formula: L/N ratio = mean counts per pixel of right lesion ROI/mean counts per pixel of contralateral normal area. To assess the therapeutic responses, we calculated the percentage change of L/N ratios according to the following formula: % change of L/N ratio = (L/N ratio – baseline L/N ratio) / baseline L/N ratio. The L/N ratio on day 1 was set as the baseline. The average radioactivity level within the infarct was obtained from the mean pixel values, normalized to that of non-ischemic cortex, and expressed as a percentage.

### Immunohistochemical Staining

Immunohistochemical investigation was done to determine the potential effect of bevacizumab on angiogenesis. At the end of week 4, the rats were deeply anesthetized after the last PET scan and perfused transcardially with 0.9% saline followed by 4% ice-chilled paraformaldehyde in PBS (pH 7.4). The brain was immediately removed, sliced, and immersed in the same fixative for 24 h. After that, the 5–10 mm slices were washed in $dH_2O$ for 30 min, dehydrated in ascending ethanols, cleared in xylene, and embedded in paraffin wax. Serial sections were cut at 4 μm throughout the ischemic area, and stained using the EnVisionTM two-step protocol with high-temperature antigen retrieval. The slides were incubated with 3% $H_2O_2$ for 10 min and rinsed 3 times in PBS for 5 min. Sections were permeabilized with 0.04% Triton X-100, blocked with 10% normal goat serum in PBS for 1 h, and incubated overnight in a humidified chamber at 4°C with the primary antibodies rabbit polyclonal antibody against VEGF (1:400 dilution; EMD Millipore, Billerica, MA) and rabbit polyclonal antibody against von Willebrand factor (vWF) (1:200 dilution; DAKO, Glostrup, Denmark). The sections were rinsed 3 times with PBS for 10 min each and treated with HRP-conjugated secondary antiserum (DAKO EnVisionTM kit) for 30 min at 37°C. Then the stained sections were washed thoroughly and developed by 0.05% diaminobenzidine with 0.03% $H_2O_2$ for 3–5 min until a brown reaction product was observed. The number of cells positively-stained for vWF in 5 microscope fields (469 μm × 351 μm) was counted. Hotspots on the section were selected at ×100 magnification in order to evaluate the average integrated optical density (IOD) of VEGF. Individual measurements were then made at ×200 (BX60, Olympus, Japan). Images (5 fields/section, 1 section/animal) were digitized with a camera. The IOD values for VEGF were obtained using Image-ProPlus 5.0 software (Media Cybernetics, Silver Spring, MD).

### Statistical Analysis

Data are expressed as mean ± SEM. The independent sample-*t* test was used to evaluate functional recovery, the PET index, and immunohistochemically-positive cells. Statistical analyses were performed with SPSS software (version 15.0, SPSS Inc, Armonk, NY). Values of $P$ <0.05 were considered statistically significant.

## RESULTS

### Influence of Bevacizumab on Functional Recovery

No significant difference in neurological score was detected between the groups at each time point of the 4-week experimental observation (Fig. 1), suggesting that bevacizumab does not affect functional recovery.

### Changes in Glucose Metabolism

The rats were studied using $^{18}$F-FDG PET in order to document whether bevacizumab influences glucose metabolism. The scans allowed the visualization (Fig. 2) and quantification (Fig. 3) of glucose metabolism throughout the brain at each time point. Semi-quantitative measurement of $^{18}$F-FDG radioactivity in the ischemic area demonstrated no significant differences in the percentage

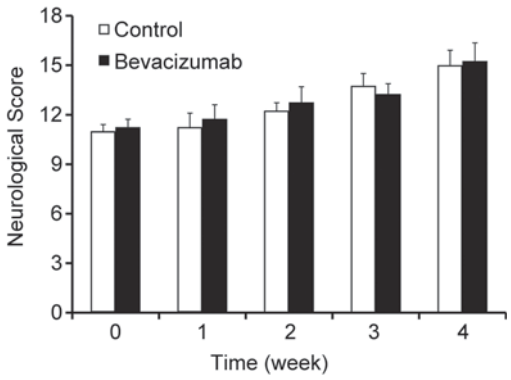


**Fig. 1. Neurological scores before and after bevacizumab treatment.**

change of the L/N ratio between the two groups on day 1 (before bevacizumab or NS injection) and at weeks 1 and 2 after MCAO. However, the percentage change in the bevacizumab-treated group was significantly lower than that in the NS group at week 3 (0.46 ± 1.15 *vs* 14.80 ± 2.33; $P$ <0.05) and week 4 (6.48 ± 1.91 *vs* 14.64 ± 0.89; $P$ <0.05) (Fig. 3), indicating that bevacizumab reduces glucose metabolism in the ischemic area.

### Immunohistochemistry

The results showed that the number of vWF-positive cells in the bevacizumab group was lower than that in the NS

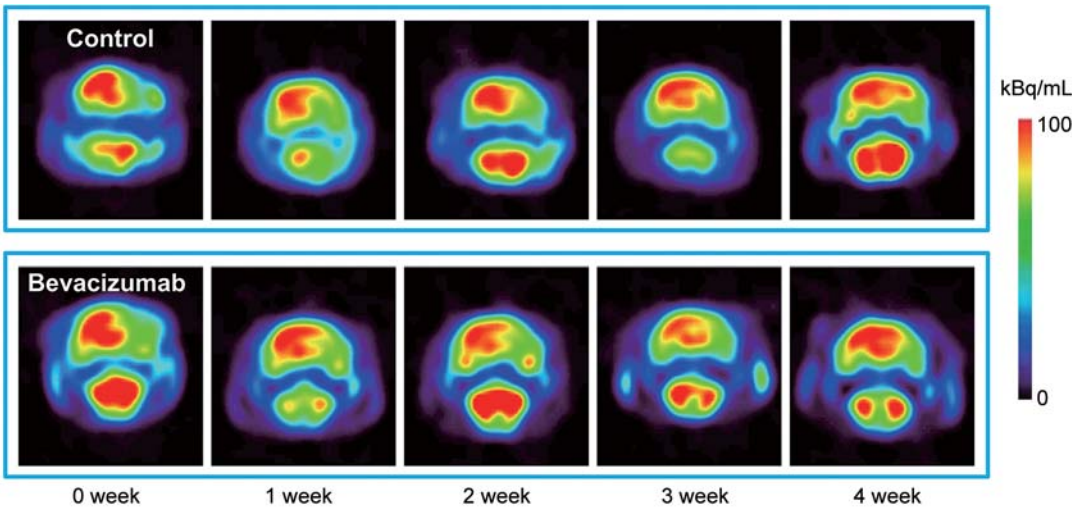


**Fig. 2. Serial PET images demonstrating metabolic recovery after MCAO in rats after bevacizumab compared to the control group. Images show the brain in coronal view. Scale indicates signal intensity.**

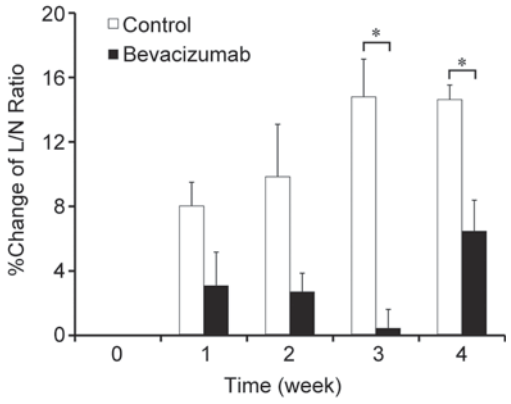


**Fig. 3. Semiquantitative analysis of variance of glucose metabolism in each group (shown as % change of L/N ratio at each time point). *$P$ <0.05.**

group (7.05 ± 0.43 *vs* 8.67 ± 0.32; $P$ <0.01) (Fig. 4). The IOD of VEGF in the bevacizumab group was higher than that in the NS group (15.22 ± 0.80 *vs* 12.83 ± 0.76; $P$ <0.05) (Fig. 4).

## DISCUSSION

Both ischemic stroke and cancer are leading causes of morbidity and mortality among the aged worldwide. Stroke severely impacts cancer patients, while cancer increases the risk of stroke[30]. CVD occurs commonly in cancer patients, ~15% of whom experience a thromboembolic event during the clinical course[5].

In the present study, we evaluated the effect of bevacizumab on neurogenic recovery after a recent stroke

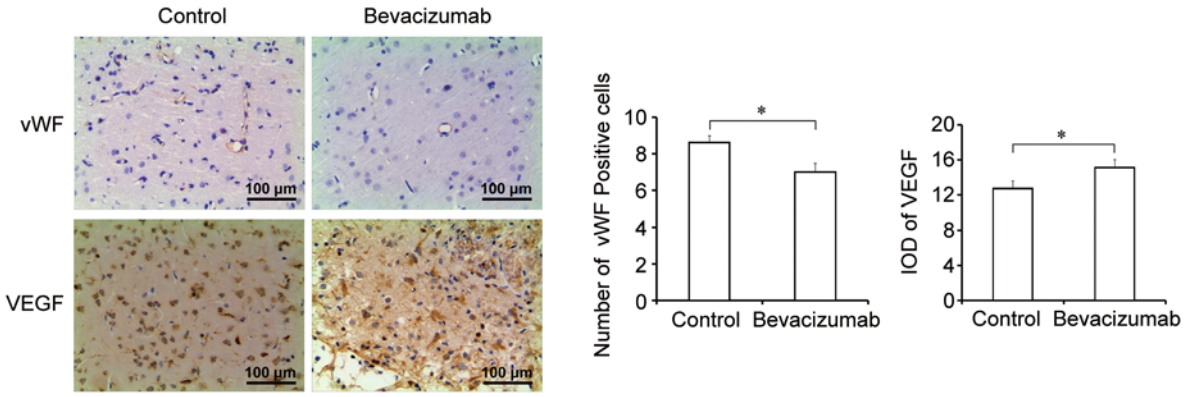


**Fig. 4. vWF and VEGF immunohistochemistry. Left, photomicrographs (×200) of immunohistochemical staining for vWF and VEGF (brown cells) in the ischemic region in bevacizumab-treated and normal saline-injected animals (control). Middle, number of vWF-positive cells after 4 weeks of bevacizumab treatment (*$P$ <0.01). Right, integrated optical density (IOD) of VEGF after 4 weeks of bevacizumab treatment (*$P$ <0.05).**

using serial $^{18}$F-FDG PET scans combined with neurologic function testing and immunohistochemical investigation. Our results demonstrated that bevacizumab had no effect on functional recovery after a recent ischemic stroke; however, it did suppress the recovery of cerebral glucose metabolism. Immunohistology confirmed a decrease in angiogenesis in the bevacizumab-treated group.

Our study showed no significant difference between the two groups in terms of glucose metabolic changes in the area of infarction during the first 2 weeks after MCAO. However, in the later stage (weeks 3 and 4), significantly lower $^{18}$F-FDG accumulation was observed in the infarct in the bevacizumab-treated group than in the control group. These results indicated that bevacizumab does not significantly inhibit metabolism immediately after MACO and in the early stage (weeks 1 and 2), but does significantly inhibit it in the later stage (weeks 3 and 4), especially at week 3. Interestingly, at week 4, the bevacizumab-treated group showed increased $^{18}$F-FDG accumulation compared to that in week 3, indicating that the inhibitory effect of bevacizumab declines over time. These results are consistent with the instructions for bevacizumab, namely, it is safer to do surgery 4 weeks after treatment.

In behavioral tests, there was no significant difference in neurological score between the two groups at any time point, suggesting that bevacizumab does not affect functional recovery. These results demonstrated that bevacizumab is relatively safe in the first 4 weeks in a rat model of cerebral ischemia.

vWF, released upon perturbation of endothelial cells, is a predictive biomarker of vascular injury. VEGF has the capacity to induce physiological and pathological angiogenesis, while bevacizumab can decrease this capacity by preventing the interaction of VEGF with its receptors on the surface of endothelial cells[31]. In the current study, immunohistochemical analysis of the injured brain showed significantly lower expression of vWF but higher expression of VEGF in the treatment group than in the control group. This result indicated that bevacizumab has a strong effect on decreasing the number of vessels, consistent with a previous study[32]. However, other cancer studies[33-34] differ from ours, in that bevacizumab was reported to reduce the high expression of VEGF. We consider that this might be due to the much lower level of VEGF expression in the ischemic brain compared to that in a tumor. In a recent study on candesartan, a drug currently used to treat hypertension, VEGF expression was found to significantly increase at 2 weeks after MCAO compared to baseline[35]. Ischemic neurons increase VEGF expression by activating astrocytes, and the increase usually occurs within the first few hours of ischemic stroke[36]. Since the endpoint of our study was 28 days after bevacizumab administration, its effect on VEGF was assumed to be decreased after its biological half-life of ~5 days[37,38].

In conclusion, the present results indicate that attention should be paid when using bevacizumab and careful management should be provided for cancer patients

with recent ischemic stroke. Although the present study was done to evaluate the safety of bevacizumab in cancer patients with recent stroke using a cerebral ischemic rat model, there are several limitations in terms of sample size, dose, investigation time points, and duration of follow-up.

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