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dl-3-*n*-Butylphthalide attenuates reperfusion-induced blood-brain barrier damage after focal cerebral ischemia in rats[1]

CHONG Zhao-Zhong, FENG Yi-Pu[2] (*Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100050, China*)

KEY WORDS butylphthalide; transient cerebral ischemia; blood-brain barrier; brain edema; Evans blue; capiliary permeability; immunohistochemistry; electron microscopy; vascular endothelium; reperfusion injury

ABSTRACT

AIM: To study the protective effect of *dl*-3-*n*-butylphthalide (NBP) on blood-brain barrier (BBB) damage induced by reperfusion following focal cerebral ischemia. **METHODS**: Focal cerebral ischemia in rats was performed by inserting a nylon suture into intracranial segment of internal carotid artery to block the origin of middle cerebral artery and reperfusion by withdrawing the nylon suture. Permeability of BBB was determined by extravasation of the protein-bound Evans blue dye to cerebral cortex and further evaluated by immunohistochemical or electronmicroscopic method. **RESULTS**: Reperfusion for 3 h following focal cerebral ischemia for 3 h produced BBB damage which exhibited the increase in extravasation in cerebral cortex, elevation of the expression of immunoglobulin (IgG), and pore formation in endothelial cell membrane of capillary in cerebral cortex. NBP (5 - 20 mg·kg^{-1}) decreased the extravasation in a dose-dependent manner. The expression of IgG in cerebral cortex was decreased and the ultrastructure damage of capillaries was alleviated after treatment with NBP. NBP 20 mg·kg^{-1} also alleviated brain edema caused by 3-h reperfusion following 3-h middle cerebral artery occlusion (MCAO). **CONCLUSION**: NBP has protective effect on BBB damage induced by reperfusion after MCAO.

INTRODUCTION

Reperfusion after focal cerebral ischemia leads to blood-brain barrier (BBB) disruption[1-3], which may exacerbate ischemic brain injury. Therefore, to protect the integrity of BBB is important for treating cerebral ischemia and reperfusion. *dl*-3-*n*-Butylphthalide (NBP), a newly developing anti-cerebral ischemic drug, has attenuating effects on cerebral ischemic damage[4-6]. In this experiment, we studied the effect of NBP on BBB permeability after focal cerebral ischemia and reperfusion in rats.

MATERIALS AND METHODS

Agents NBP, a yellow oil, was first isolated from the seeds of celery in Europe and synthesized by Department of Medicinal Synthetic Chemistry of our Institute, was made into emulsion with Tween 80.

Middle cerebral artery occlusion (MCAO) Wistar rats, ♂, 285 g ± *s* 21 g, Grade Ⅱ, Certificate № 01-3008, were anesthetized with 10 % chloral hydrate (400 mg·kg^{-1}, ip). Room temperature was maintained at 24 - 25 ℃. The right common carotid artery, external (ECA), and internal carotid artery (ICA) were exposed through a ventral middle incision in the neck. A nylon suture (ø 0.28 mm) was inserted into ICA from ECA and put forward about 20 mm from the origin of ICA into the intracranial segment of ICA to block the origin of middle cerebral artery (MCA). The rats which showed ischemic behavior (failure to extend left forelimb fully) 170 min after the onset of ischemia were used. For reperfusion 180 min after ischemia, the nylon suture was pulled out to restore the blood flow to MCA.

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[2] Correspondence to Prof FENG Yi-Pu. Phn 86-10-6316-5173. Fax 86-10-6316-7757. E-mail fengyp@public.gb.com.cn
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Determination of permeability of BBB Two mL·kg^{-1} of 25 g·L^{-1} Evans blue in normal saline was injected through sublingual vein 5 h after the onset of MCAO. One h later, the rats were perfused with saline 60 mL through left ventricle to clear the dye from brain vasculature. The Evans blue dye in ipsilateral or contralateral cortex was extracted in formanide 3 mL at 24 – 25 ℃ for 72 h. The dye was quantified fluorometrically (Hitachi MPF-4 fluorescence spectrophotometer, λ_{ex} = 625 nm, λ_{em} = 675 nm, slit = 18 nm). The cerebral cortex was dried at 100 ℃ for 3 d and calculated for water content.

Experimental protocols The rats were divided into 6 groups: (A) Sham group received an exposure of carotid arteries without insertion of nylon suture, n = 5; (B) Vehicle 1 (permanent MCAO, pMCAO) group subjected to a 6-h permanent MCAO, n = 7; (C) Vehicle 2 (temporary MCAO, tMCAO) group subjected to 3-h reperfusion following a 3-h MCAO, n = 7; (D) NBP 5 mg·kg^{-1} group, n = 6; (E) NBP 10 mg·kg^{-1} group, n = 6; (F) NBP 20 mg·kg^{-1} group, n = 6. Groups D – F subjected to tMCAO and NBP ip 5 min and 60 min after the onset of ischemia.

Immunohistochemistry After a 3-h reperfusion following a 3-h cerebral ischemia, the serum proteins in the vascular space were flushed out by perfusion with saline 60 mL followed by 4 % paraformaldehyde 60 mL through left cardiac ventricle. The brain was placed in 4 % paraformaldehyde for further fixation. Paraffin-sections (15 μm) were used. Anti-serum protein IgG immunohistochemical was carried out using streptavidin/peroxidase immunohitochemical analysis kit (Beijing Zhongshan Biotechnological Co).

Electron microscopy At the termination of reperfusion, cerebral cortex which was cut into 1 mm^3 was fixed in 2.5 % glutaraldehyde for 2 h. After rinsing in Na^+-cacodylate 0.1 mol·L^{-1} buffer (pH 7.4), the tissue was post-fixated in 1 % OsO_4 for 1.5 h, dehydrated in a series of graded propanone and embedded in araldite. Ultrathin sections (50 nm) were viewed under H-800 electron microcope.

Statistical analysis The Data were expressed as $\bar{x} \pm s$ and compared by t test.

RESULTS

Effects on Evans blue and water content Reperfusion induced an increase in BBB permeability. The Evans blue content in ischemic cortex was 3 times than that in contralateral cortex ($P < 0.01$), indicating that BBB was injured by reperfusion after MCAO. However, there was no change in BBB permeability in ischemic cortex compared with contralateral cortex in pMCAO group (Tab 1).

Brain edema in ischemic cortex was induced by a 3-h reperfusion following a 3-h MCAO. The water content in ischemic cortex in vehicle group (81.7 % ± 1.9 %) was increased compared with that in contralateral cortex (79.4 % ± 1.7 %). Six-h pMCAO also slightly induced an increase in water content in ipsilateral cortex ($P > 0.05$).

The content of Evans blue was decreased by 33.3 %, 46.7 %, and 50.7 %, respectively at the dosage of 5, 10, and 20 mg·kg^{-1}. The water content was also decreased in ischemic cortex after treatment

Tab 1. Permeability of blood-brain barrier to protein-bound Evans blue dye and brain water content in contralateral and ischemic cortices after a 6-h permanent middle cerebral artery occlusion (pMCAO), 3 h of occlusion plus 3 h of reperfusion (tMCAO). $x \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* contralateral cortex. $^eP < 0.05$, $^fP < 0.01$ *vs* vehicle 2 group.

Group	Dosage/ mg·kg^{-1}	Insult	Evans blue/μg·g^{-1}		Water content/%		n
			Contralateral	Ipsilateral	Contralateral	Ipsilateral	
Sham			3.7±1.8	4.1±1.2	79.0±1.5	79.4±0.9	5
Vehicle 1	0	pMCAO	2.1±0.9	3.3±0.9	79.1±0.9	80.6±0.9	7
Vehicle 2	0	tMCAO	5.2±1.3	16.5±4.6^c	79.4±1.7	81.7±1.9^b	6
NBP	5	tMCAO	5.2±1.4	11.0±5.8^e	78.3±1.2	80.2±2.6	6
	10	tMCAO	5.3±1.5	8.8±2.8^f	79.3±1.6	80.7±1.8	6
	20	tMCAO	6.0±1.9	8.14±2.0^f	79.5±1.2	79.6±1.8^e	6

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with NBP (20 mg·kg^{-1}) compared with ipilateral cortex in vehicle group. The Evans blue and water content showed no significant change in contralateral cortex in tMCAO, pMCAO, and NBP treated groups (Tab 1).

Effect on the expression of IgG Reperfusion induced an obvious increase in the expression of IgG in the ischemic brain tissue, further displayed the increase in permeability of BBB to serum protein. NBP (10 – 20 mg·kg^{-1}), given 5 and 60 min after the onset of cerebral ischemia, appeared to decrease the quantity of IgG expression (Fig 1).

Effect on ultrastructure of capillary Pores formed in the endothelial cell membrane and mitochondria damage (vacuolated) after reperfusion. Tissue edema was observed around the capillary. The above damage was alleviated after treatment with NBP (10 – 20 mg·kg^{-1}) (Fig 2).

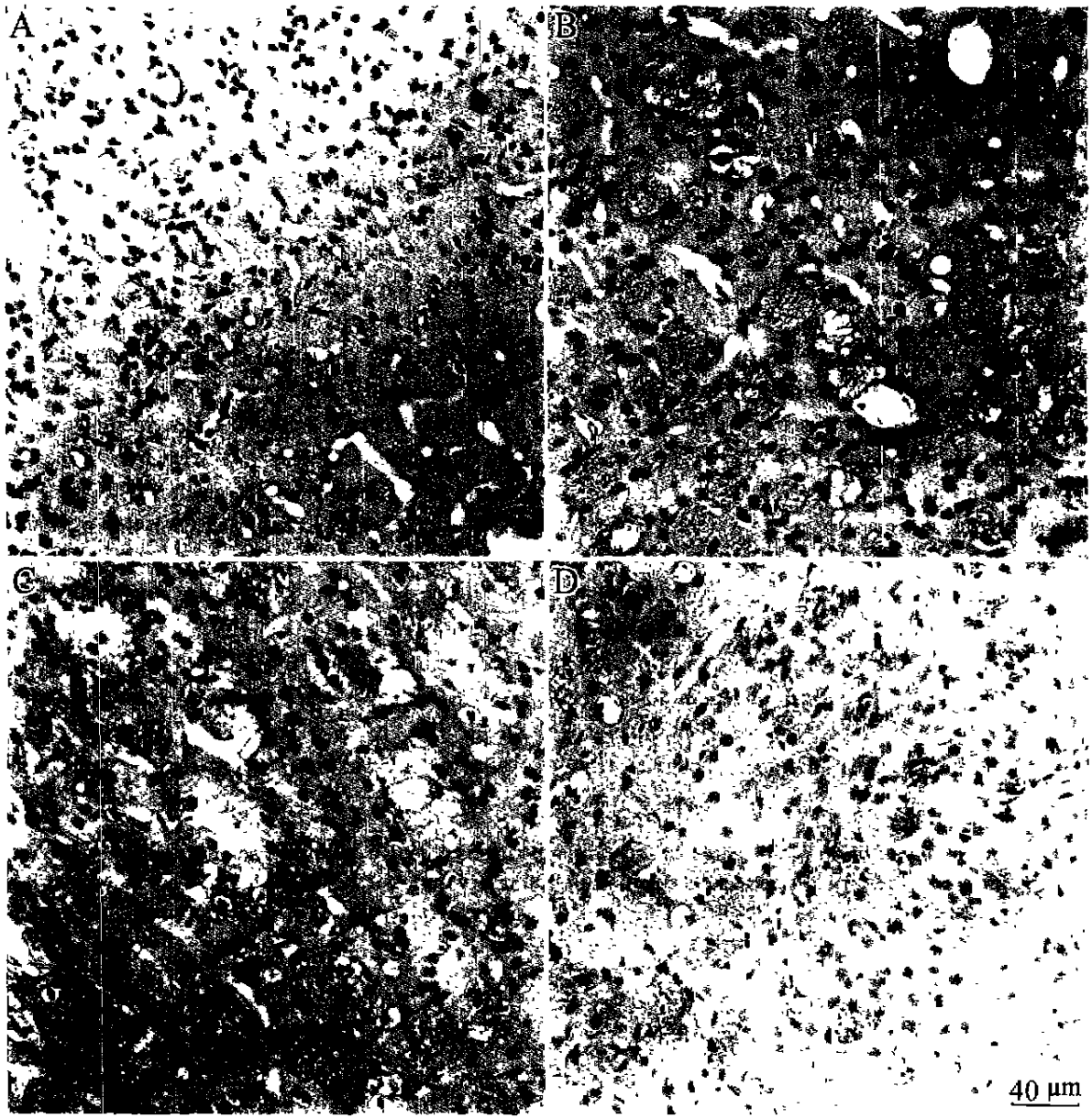

Fig 1. Immunohistochemical staining for IgG in cerebral cortex (5 × 50) of rats subjected to 3-h reperfusion following 3-h focal cerebral ischemia. $n = 3$ rats. A) Sham. B) Vehicle group. C) NBP 10 mg·kg^{-1}. D) NBP 20 mg·kg^{-1}.

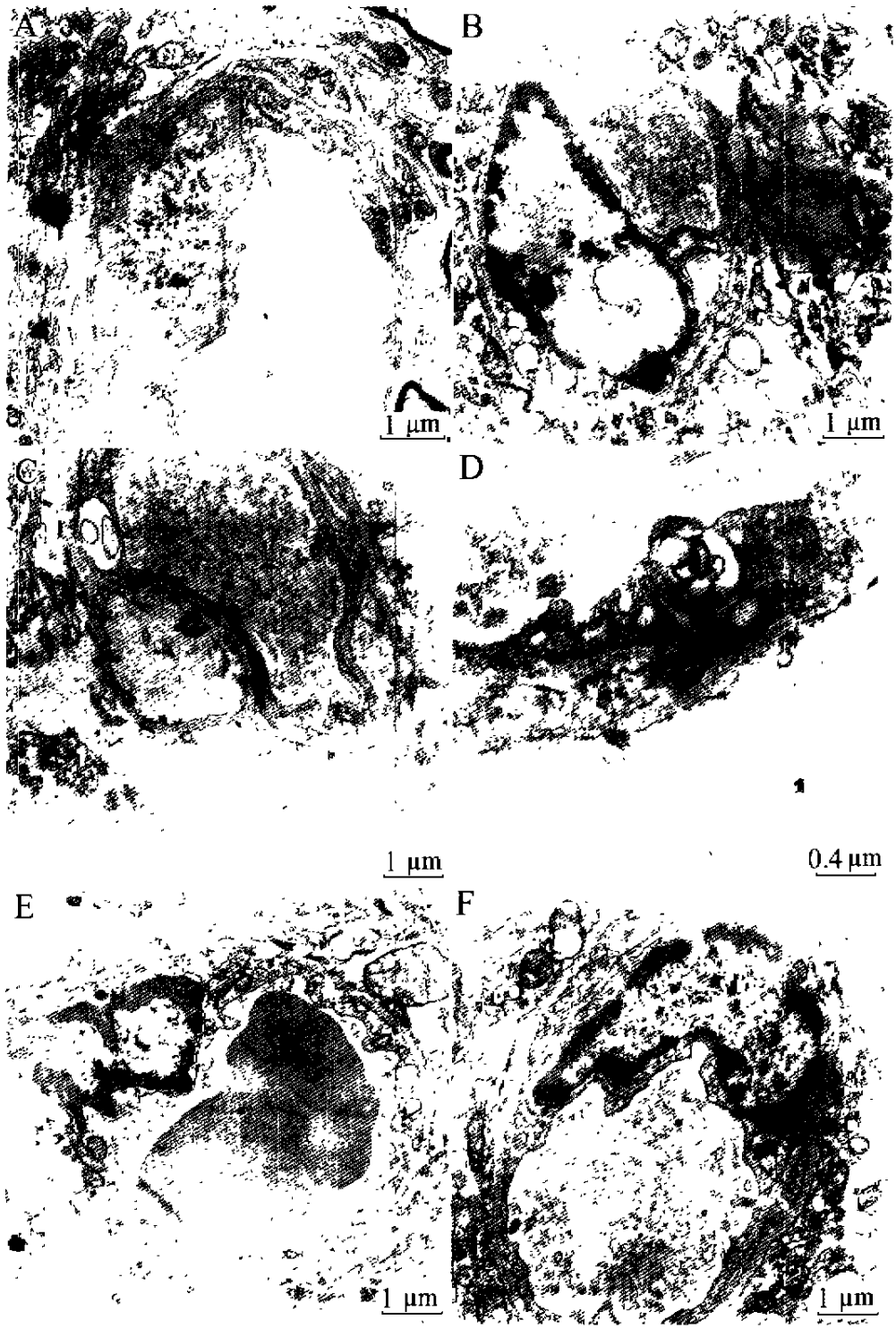

Fig 2. Ultrastructure of cerebral (stained with Pb acetate and Pb citrate) microvessel in ischemic cerebral cortex of rats subjected to 3-h reperfusion. A) Contralateral to ischemic cortex (× 12 000). B – D) Vehicle group, ipsilateral cortex (B – C: × 12 000, D: × 30 000). E) Treated with NBP 10 mg·kg^{-1}(× 12 000). (F) Treated with NBP 20 mg·kg^{-1}(× 12 000).

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DISCUSSION

Our results indicated that BBB permeability to serum protein bound Evans blue was maintained integrity after a 6-h pMCAO and was disrupted after a 3-h reperfusion following a 3-h MCAO. These results are consistent with previous report[3]. The ultrastructure damage such as pore formation in cell membrane and mitochondria vacuolation in endothelial cells of capillary in cerebral tissue may be one of main reasons of the increase in permeability of BBB after reperfusion following cerebral ischemia.

One of possible mechanisms of reperfusion-induced BBB damage after ischemia is the production of oxygen free radical, which may cause cell membrane damage. NBP has been reported to inhibit the elevation of hypoxanthine and xanthine which increase free radicals when they change into xanthine and uric acid by xanthine oxidase during reperfusion after four-vessel occluded ischemia[6]. Therefore, the protective effect of NBP may be related to its action on purine metabolism. NBP also increased regional cerebral blood flow during focal cerebral ischemia in rats and thus may reduce reperfusion injury. The effect might contribute to its protective effect on BBB.

NBP had been proved to reduce brain edema after 24-h MCAO through decreasing the sodium content and increasing the potassium level in brain[7]. The results of this experiment suggest that NBP may also be effective in reducing the brain edema induced by reperfusion after MCAO, the mechanism of which may be partly related to its protective effect on BBB.

In conclusion, NBP has protective effect on BBB and this may be beneficial in the treatment of cerebral ischemia and reperfusion.

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丁基苯酞减轻大鼠局灶性脑缺血再灌注引起的血脑屏障损伤[1]

种兆忠，冯亦璞[2]（中国医学科学院，中国协和医科大学药物研究所，北京 100050，中国）

关键词 丁基苯酞；暂时性脑缺血；血脑屏障；脑水肿；伊文氏蓝；毛细血管通透性；免疫组织化学；电子显微镜检查；血管内皮；再灌注损伤

目的：研究丁基苯酞(NBP)对局灶性脑缺血再灌注引起的血脑屏障(BBB)破坏的保护作用. **方法**：尼龙线栓塞法造成大鼠局灶性脑缺血模型，尼龙线抽出进行再灌. BBB 的通透性以结合有伊文氏蓝染料的血浆蛋白外渗进入脑组织的量以及免疫组化和电镜方法进行了测定. **结果**：脑缺血 3 h 再灌注 3 h 可使结合有血浆蛋白的伊文氏蓝在缺血皮层含量明显增加；免疫球蛋白 IgG 在大脑皮层中的表达明显增加；皮层组织中毛细血管内皮细胞膜上有微小孔洞的形成，内皮细胞中线粒体空泡化. NBP 5, 10, 和 20 $mg \cdot kg^{-1}$ 可使皮层中染料含量分别下降 33.3%，46.7% 和 50.7%；缺血皮层中 IgG 的表达减少；毛细血管超微结构的损伤也有一定程度的减轻. NBP20 $mg \cdot kg^{-1}$ 亦可使脑缺血再灌后脑水肿程度明显减轻. **结论**：NBP 可减轻局灶性脑缺血再灌引起的 BBB 损伤.

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