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2-(1-Hydroxypentyl)-benzoate Increases Cerebral Blood Flow and Reduces Infarct Volume in Rats Model of Transient Focal Cerebral Ischemia

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Effects of 2-(1-Hydroxypentyl)-benzoate in Cerebral Ischemia

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Abbreviations: dl-PHPB, 2-(1-hydroxypentyl)-benzoate; dl-NBP, 3-n-butylphthalide; MCAO, middle cerebral artery occlusion; rCBF, regional cerebral blood flow; LDF, laser-doppler flowmetry; MABP, mean arterial blood pressure; CCA, common carotid artery; ECA, external carotid artery; ICA, internal carotid artery; TTC, 2,3,5-triphenyltetrazolium chloride; RP-HPLC, Reversed-phase high performance liquid chromatography.

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Abstract

2-(1-hydroxypentyl)-benzoate (dl-PHPB), a derivate of 3-n-butylphthalide (dl-NBP), is a novel drug candidate used for treatment of cerebral ischemia. The goal of the present study was to investigate the effects of dl-PHPB on infarct volume, neurological function, and cerebral blood flow (CBF) in transient focal cerebral ischemia. Therefore, an animal model of 2-hour middle cerebral artery occlusion (MCAO) followed by 24-hour reperfusion was used. Rats received dl-PHPB (1.3, 3.9 or 12.9 mg/kg) intravenously 10 min after the onset of MCAO. Compared with vehicle-control group (37.4%), infarct volume in dl-PHPB-treated groups reduced significantly and dose-dependently to 25.4%, 17.4% and 13.7%, respectively. The changes in neurological deficient were also observed in neurobehavioral test in a dose-dependent manner, and the neuronal score was improved significantly from the vehicle-control of 3.2 to 2.7, 2.1 and 1.8, respectively. At the highest dose, the potency of dl-PHPB was similar to those of dl-NBP. CBF was quantified by using laser-Doppler flowmetry. During the ischemia, the regional CBF values of dl-PHPB groups were significantly higher than that of vehicle group. In addition, our study showed that dl-PHPB converted into dl-NBP very fast in blood in vitro. About 70% of dl-PHPB converted into dl-NBP in 5 minutes when dl-PHPB was added into plasma at final concentration of 6, 30 and 60μg/ml. This result demonstrated that the neuronal protection effects of dl-PHPB was mainly induced by dl-NBP, an active compound converted from its precursor, dl-PHPB. In conclusion, dl-PHPB can reduce infarct volume and improve neurobehavioral deficits in a rat model of transient MCAO. Those effects may partially due to increase in CBF by the active metabolite (dl-NBP) of dl-PHPB. Therefore, our results suggest that dl-PHPB may be useful for treatment of ischemia stroke.

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Introduction

Stroke is the third leading cause of death and one of the leading causes of adult disability in North America, Europe and Asia (Higashida et al., 2003). Ischemic stroke occurs in 60% to 70% of all forms of stroke patients (Feng, 1999; Silvestrelli et al., 2002; Mahajan et al., 2004).

Ischemic stroke is initiated by a transient or permanent reduction in cerebral blood flow (CBF) that is restricted to the territory of major brain artery, such as the occlusion of middle cerebral artery (MCAO). MCAO impairs irrigation of focal brain areas, which show gradual reductions of CBF from the periphery to the core of the MCA territory (Bolander et al., 1989). The development of ischemic brain damage depends on the reduction of CBF below critical threshold level (Memezawa et al., 1992; Takagi et al., 1995). Then, the decrease of CBF in ischemic regions may result in an energy failure and further lead to an activation of the toxic intracellular pathway (Jorgensen et al., 1982; Dirnagl et al., 1999; Hou and Macmanus, 2002). The infarct volume is also dependent on the duration of ischemia (Soriano et al., 1997). Therefore, the severity of ischemia has two components: degree of CBF reduction and duration of the ischemic episode.

Potassium 2-(1-hydroxypentyl)-benzoate (dl-PHPB), derivated from 3-n-butylphthalide (dl-NBP), is a newly synthesized compound that is under development as a therapeutic drug for cerebral ischemia (Yang et al., 2002). As reported, dl-NBP is a primary naphtha component from seeds of *Apium graveolens Linn*. The phase 3 clinic trail of dl-NBP has been completed and it was approved by the SFDA of China at the end of 2002 as a new drug for treatment of ischemic stroke in clinic. Many basic and clinic studies have proved that dl-NBP is a potentially beneficial and promising drug for

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treatment of ischemic stroke with multiple actions which affect some pathophysiological processes, such as improving microcirculation of rat brain, inhibition of platelet aggregation, regulation of energy metabolism, inhibition of ischemia induced oxidative damage and neuron apoptosis (Chong and Feng, 1997; Yan et al., 1998; Xu and Feng, 2000, 2001; Dong and Feng, 2002). However, dl-NBP is difficult to use intravenously due to its hydrophobicity. Thus, dl-NBP is limited to use in the clinic for serious patients of ischemic stroke. Therefore, dl-PHPB was designed as a pre-drug of dl-NBP. The properties of dl-PHPB has been greatly improved. In addition, in our previous research, it was found that dl-PHPB could quickly convert to dl-NBP in vitro and in vivo (given to rat orally or intravenously). Recent it was also found that dl-PHPB could inhibit platelet aggregation *ex vivo* and reduce thrombus formation in the arteriovenous shunt model *in vivo* (Zhang et al., 2004). In the present study, the effects of dl-PHPB on CBF, infarct volume, and neurological function were investigated in a rat model of transient focal cerebral ischemia.

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Materials and Methods

Animals and Drugs. For all experiments, male Sprague-Dawley rats (weight, 270-330 g) were used. Animals were allowed free access to food and water in a temperature-controlled environment at 25 °C before and after surgery. The experiments were performed in accordance with the guidelines for the care and use of laboratory animals and were approved by the Animal Care Committee of the Peking Union Medical College and the Chinese Academy of Medical Sciences.

dl-PHPB and dl-NBP were offered by department of synthetic pharmaceutical chemistry of our institute, purity of 99.9 %. The chemical structures of these compounds are shown in Figure 1. According to the molecular weight of two compounds, doses of dl-PHPB (1.3, 3.9, 12.9 mg/kg) used to treat intravenously were equalmolar to the doses of dl-NBP (1, 3, 10 mg/kg), respectively. dl-PHPB was dissolved in 0.9% saline. dl-NBP was dissolved in component solvent (PEG_{400}:H_2O=1:3) (i.v.).

Animal Preparation and Experimental Model. After administration of atropine sulfate (0.5 mg/kg, i.p.) to minimize bronchial secretions, the rats were anesthetized with 3% isoflurane in 40% oxygen and 60% nitrogen, orally intubated, and mechanically ventilated. Temperature probe was inserted into the rectum, and separate heating lamp was used to maintain rectal temperature at normothermic level. The right femoral artery and vein were cannulated for measurement of arterial blood gases, glucose, pH, hematocrit, mean arterial blood pressure (MABP) and drug infusion. These physiological parameters were monitored before, during, and after MCAO. The rectal temperatures were still measured at 12 and 24 h after MCAO in some of animals.

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The principles and technical details of Laser-Doppler flowmetry (LDF) have been described elsewhere [Dirnagl et al., 1989]. For the continuous measurement of local regional CBF (rCBF), the rat was fixed in a stereotaxic frame. The skull was exposed and a burr hole in a diameter of 2 mm was drilled on the right side of the skull at 2 mm posterior and 3 mm lateral to the bregma (Paxinos and Watson, 1986) to accommodate the probeholder. The field was frequently irrigated with physiological saline and the dura mater was kept intact to prevent injury to the cortex. A laser-Doppler probe (Probe 407–1, Perimed, Jarfalla, Sweden), held in the probeholder, was placed perpendicularly (avoiding large blood vessels).

MCAO was induced as described by Longa et al (1989) and Belvyev et al (1996), and then modified by us. After a midline neck skin incision, the right common carotid artery (CCA) and bifurcation was exposed and the external carotid artery (ECA) and the internal carotid artery (ICA) were dissected free from surrounding nerves and fascia. The ECA were ligated distally and a 4-0 nylon suture (its tip rounded by heating and coated with poly-L-lysine) was introduced into the lumen of the ICA through a small incision on the stump of the ECA. The suture was gently advanced into the ICA for 19-20 mm from the bifurcation of CCA to block the origin of MCA, and the ipsilateral laser–Doppler signal decreased to approximately 20%-30% of baseline. After 2 h of MCAO, reperfusion was achieved by withdrawal of the intraluminal suture from ECA. At 50 min of reperfusion, the Laser–Doppler flow probe and catheter were removed. The trachea was extubated and the neck incision was closed with silk sutures. The animal was allowed to awaken from anesthesia and survive for 24 h after ischemia.

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Treatment Groups and Drug Administration. Rats were divided into six experimental groups randomly: three dl-PHPB treated groups (1.3, 3.9, 12.9 mg/kg), one dl-NBP treated groups (10 mg/kg), one vehicle-control group and one sham-operate group. At 10 min after the onset of MCAO, drugs or vehicle were administered intravenously to rats in a volume of 1 ml/kg body weight.

Regional CBF Measurement. Regional CBF (rCBF) was measured continuously before, during the acute phase of ischemia and after reperfusion by means of a LDF. The rCBF value was calculated as the average value during a period of every 10 min. Firstly, the rCBF was monitored for 10 min prior to MCAO to establish the baseline value (100%). Then MCAO was induced by advancing the filament. After 2 h of occlusion, the filament was withdrawn. The changes in rCBF were expressed as a percentage of the baseline value.

Neurobehavioral Testing. After surgery, each animal's neurological function was evaluated at 24 h of reperfusion. Neurological deficit was graded on a score of 0 to 4 as previously described (Menzies et al., 1992; Belvyev et al., 1996; Mokudai et al., 2000): 0, no observable deficit; 1, forelimb flexion; 2, forelimb flexion and decreased resistance to lateral push; 3, forelimb flexion, decreased resistance to lateral push, and unilateral circling; and 4, forelimb flexion and being unable or difficult to ambulate.

Infarct Assessment. At 24 h of reperfusion, the animals were anesthetized with Trichloroacetaldehyde monohydrate(350 mg/kg, i.p.) and decapitated. The brain was then rapidly removed, cut into six 2-mm-thick coronal sections (the last section was

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4-mm-thick) by use of a rat brain matrix (RBM 4000C, ASI Instruments), stained with 4% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma Co., USA) at 37℃ for 30 minutes. Infarct area on each slice was determined by using digital imaging with a computerized image analyzer (SPOT 3.5 Biometrics software), and the infarct areas were calculated to obtain the infarct volumes per brain (in mm3). Infarct volumes were expressed as a percentage of the contralateral hemisphere volume by using an "indirect method" (area of intact contralateral [left] hemisphere minus area of intact regions of the ipsilateral [right] hemisphere) to compensate for edema formation in the ipsilateral hemisphere (Swanson et al. 1990; Mokudai et al. 2000).

$$I\ \% = (Vc - Vi) / Vc \times 100\%$$

Vc = volume of intact contralateral [left] hemisphere

Vi = volume of intact regions of the ipsilateral [right] hemisphere

Conversion of dl-PHPB in vitro. dl-PHPB was added into rat plasma at the final concentration of 6, 30 and 60μg/ml, and incubated for different periods (2, 5, 10 and 20 min). After the incubation, 200μl of methanol was added into 100μl the plasma sample. Then the samples were mixed by vortex for 20 min and centrifuged for 10 min at 8000 rpm. The supernatant was collected and directly injected into the HPLC (Agilent 1100) system. For quantification of dl-PHPB and dl-NBP, Aichrom Bond-1 ODS column (particle size 5μm, 4.6×250mm，pH2~10, USA) and the mobile phase comprised of methanol-0.1% phosphate buffer (50/50 for dl-PHPB, 65/45 for dl-NBP) were used. The flow rate was 1 ml/min. The wave length for UV detection was 210nm. The column temperature was kept at 25±0.5℃. 4-Biphenylacetic acid (10 μl, 0.5mg/ml) and

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diazepam (10 μl, 0.5mg/ml) were used as the internal standard for quantification of dl-PHPB and dl-NBP, respectively.

Statistical Analysis. The results are expressed as mean±S.E.M. Physiological data, neurological score and infarct volume were statistically analyzed using one-way ANOVA followed by Dunnet's test for comparing the treatment groups and vehicle-control group. The data from rCBF were analyzed by two-way ANOVA to detect difference between groups and over time. Result was considered to show a significant different when P value smaller than 0.05.

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Results

Physiological Parameters Before and After Brain Ischemia.

The physiological parameters for all groups are provided in Table 1. All data were kept within normal physiological limits before, during, and after ischemia. There were no significant differences in arterial blood gases, glucose, pH, hematocrit, and MABP between the vehicle and dl-PHPB treated groups.

The rectal temperature in all groups was regularly maintained at normal level (37℃) during surgical procedures (before, during ischemia, and 30 min of postischemia) under conditions of anesthesia and temperature-controlled system, as shown in Table 1. At later reperfusion time points (12 and 24 h of postischemia) after rats recovered from anesthesia, the temperature increased definitely and reached about 39℃ in vehicle group, as well as in drug-treated groups. But there were no significant differences between them.

Neurological Assessment.

Before MCAO, neurological score was zero in all animals. After MCAO, high-grade neurological deficits were present. The results are shown in Table 2. Compared with vehicle-treated rats (score=3.2 ± 0.2), treatment with dl-PHPB significantly and dose-dependently improved the neurological score at 24 h after ischemia. At the highest dose, the efficacy of dl-PHPB (score=1.8±0.2) was similar to dl-NBP (score=2.0±0.2).

Infarct Volume And Protective Effect of dl-PHPB.

Coronal sections of rat brain, stained with TTC, are shown in Figure 2. Infarct volume

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was measured in animals following transient MCAO after treatment with dl-PHPB, dl-NBP or vehicle (Figure 3). At doses of 1.3, 3.9, 12.9 mg/kg, dl-PHPB could dose-dependently reduce the infarct volume significantly from a vehicle-control of 37.4% to 25.4%, 17.4% and 13.7%, respectively ($P<0.01$ vs. vehicle-control). The infarct volume in dl-NBP group (10 mg/kg) was 16.3%. This indicates that dl-PHPB show the similar efficacy with dl-NBP in the reduction of infarct volume at the corresponding dose.

Effects of dl-PHPB on regional CBF

Figure 4 shows the relative changes of rCBF with LDF during and following 2 h of MCAO. In all groups, MCA occlusion induced a similar immediate reduction of rCBF value to about 20-30% of the baseline level. During the ischemic period, the rCBF values in dl-PHPB groups and dl-NBP group were significantly higher than that in vehicle group. Especially at high dose, dl-PHPB increased rCBF significantly from 20 min after administration and throughout the ischemic period. The potency of dl-PHPB was a little bit stronger than that of dl-NBP, but there is no significant difference between them at the corresponding dose. After recirculation, rCBF gradually recovered to about 70-80% of the baseline level. At the 50 minutes reperfusion, there were no significant differences between the vehicle and dl-PHPB groups.

Two-way ANOVA of data, obtained after injection of dl-PHPB and dl-NBP, showed a significant effect of treatment ($P<0.05$ vs. vehicle-control), but no effect of time during the ischemia.

Conversion of dl-PHPB to dl-NBP in vitro.

Using our analysis system the rate of recovery from rat plasma was 98.3% for dl-PHPB

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and 84.7% for dl-NBP, respectively. It is showed in Figure 5. After adding into plasma, the levels of dl-PHPB decreased very fast, and the levels of dl-NBP in plasma simultaneously increased. At 5 min incubated dl-PHPB with plasma, more than 70% of dl-PHPB was converted into dl-NBP. These indicated that dl-PHPB could convert into dl-NBP fast and completely at concentration of 6, 30 and 60μg/ml.

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Discussion

The ischemia-induced changes and functional impairments after permanent or transient occlusion of the MCA in rats closely resemble those observed after focal occlusion of the MCA in humans (Yamori et al., 1976), resulting in the frequent use of this model for evaluating neuroprotective agents (Muller et al., 1995; Engelhorn et al., 2004). So in present study, we used a rat model of 2-hours intraluminal suture occlusion of MCA and 24-hours reperfusion to produce transient focal cerebral ischemia.

As describe above, the severity of damage after transient cerebral ischemia depends mainly on the duration of the ischemia and the degree of CBF reduction. For a given period of ischemia (2-hours MCAO in our study), the infarct volume will be function of CBF reduction. Yet, CBF in the ischemic core is usually strongly affected for quite a wide range of infarct sizes. Therefore, CBF in the periphery of the affected area (penumbra) can potentially indicate the extent of CBF alterations and, therefore, might give an estimation of the severity of ischemia (Memezawa et al., 1992; Soriano et al., 1997). Many kinds of methods are widely used for measuring CBF. All methods have strengths and weaknesses (Kramer et al., 1996). Compared with other methods, LDF provides a noninvasive and continuous measure of local CBF, increasing the ability to observe instantaneous changes in cerebral microcirculation. The changes of focal CBF after MCAO can be monitored by project zones of MCA in the surface of cerebra with LDF, which seems to give good estimations of CBF reductions. However, LDF did not record absolute blood flow, and it was very sensitive to an altered position of LDF probe. In addition, under conditions of changed blood hematocrit or fluctuations of blood gases, LDF did not accurately measure CBF values (Kramer et al., 1996; Gu et al., 2003). So,

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physiology parameters should be monitored at the same time.

dl-NBP was demonstrated a useful drug for treatment of ischemic stroke in clinic studies. But it was limited to use intravenously due to its property of hydrophobicity, and it was difficult in treatment of serious patients. Therefore, dl-PHPB was designed as a pro-drug of dl-NBP and its properties have been greatly improved. In this study, it was shown that about 70% of dl-PHPB could convert into dl-NBP in 5 min, when dl-PHPB was added into the plasma in vitro. Pharmacokinetic study in vivo also showed that dl-PHPB converted into dl-NBP fast and completely after orally or intravenously administered. When given dl-PHPB intravenously to rat, it was difficult to be determined in the plasma. However, after dl-PHPB injection 1 min, dl-NBP was found clearly in rat plasma with quite high level. (unpublished data). The present results showed that intravenous treatment of dl-PHPB (1.3, 3.9, 12.9 mg/kg) could dose-dependently reduce the infarct volume significantly. And at the highest dose, dl-PHPB showed slightly higher efficacy in reducing infarct volume than dl-NBP, although there was no significant difference between two compounds.

Neurobehavioral deficits were also observed 24-hours after MCAO. It showed that dl-PHPB could also improve the behavioral deficits in a dose-dependent manner. The improvement effects of dl-PHPB observed were highly correlated with the reduction of infarct volumes at the same doses.

To determine whether the infarct-reducing effects of dl-PHPB are due to an increase in blood flow during ischemia, we measured rCBF in the penumbral region of cortex (2 mm posterior and 3 mm lateral to the bregma), which is also the project zone of MCA in the surface of cerebral. Our data showed that treatment with dl-PHPB could significantly improve rCBF during the period of occlusion, whereas the improving effect at the highest

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dose was far more potent than that at the middle or lowest dose. At the highest dose of dl-PHPB injection, CBF value increased from about 27% to 40% of the preischemia values (CBF value in the vehicle-treated group decrease from 25% to 15%). While CBF value in the middle dose group only increased slightly from about 23% to 26%, just similar as the effect in the lowest dose (from about 29% to 24%). Compared with the dose-dependent results from the infarct and neurobehavioral assessments, such results indicated that the neuroprotective effect of higher dose of dl-PHPB observed in infarct and neurobehavioral test was mainly due to an improvement of blood flow during ischemia. However at lower doses, the neuroprotection of dl-PHPB appeared to be a result of multiple mechanisms, such as antioxidative and antiapoptosis effects that have been observed in our researches same as in dl-NBP (Dong and Feng, 2002; Chang and Wang, 2003). Following withdrawal of the intraluminal suture, rCBF in all groups gradually recovered. There was also a trend that the CBF values received after reperfusion in the treated groups were obviously higher than those in vehicle group. Especially at high dose, treatment with dl-PHPB increased the CBF level by about 51.6% than vehicle group at 30 min after reperfusion, although there were no significant differences between the vehicle and the treated group at the same individual time points.

Various physiological variables influence CBF, including arterial blood gases, cerebral autoregulation, metabolic rate through vasoneuronal coupling, and functional balance between the endothelium dependent vasodilators and the endothelium derived constrictors. One important mediator controlling basal CBF is nitric oxide (NO). Endothelium derived NO plays a crucial role in maintenance of blood vessal caliber and therefore blood flow throughout the vasculature. It is also important in preventing thrombosis through inhibition of platelet adhesion, activation, and aggregation (Markus,

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2004). Arachidonate metabolism also plays important role in modulating CBF. Thromboxane A_2 (TXA_2) and prostaglandin I_2 (PGI_2) are major metabolites of cyclooxygenase activation in platelets and in endothelial cells, respectively. TXA_2 is a potent platelet aggregating and vasoconstricting substance, while PGI_2 is a powerful antiplatelet and vasodilator agent (Peng et al., 2004). Previous studies showed that dl-NBP could induce the activation of endothelial NO synthase and thus increasing the production of NO (Yan and Feng, 1998). Furthermore, dl-NBP could decrease the production of TXA2 and increase the production of PGI2 in cerebral cortex cells, and decrease the ratio of TXA2/PGI2 in brain tissue after MCAO in rat (Chong and Feng, 1997). Therefore, such activities might be also involved in the rCBF improvement effects of dl-PHPB.

In conclusion, dl-PHPB significantly reduces infarct volume and improves neurobehavioral deficits in rat model of transient focal cerebral ischemia. Such effects may partly due to the increase of rCBF. In addition, multiple mechanisms for dl-PHPB mediated neuroprotection must be involved in. Therefore, further studies are needed to completely elucidate the mechanism accounting for the protective effects of dl-PHPB on cerebral ischemia.

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Footnotes

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Legends for Figures

Fig. 1. Chemical structures of dl-PHPB and dl-NBP.

Fig. 2. Effect of dl-PHPB (i.v.) on infarct volume in rat brain after 2 hours intraluminal suture occlusion of MCA and 24 hours reperfusion. The TTC-stained coronal brain sections are from representative animals of Vehicle-control group (**A**), dl-PHPB (1.3, 3.9 and 12.9 mg/kg) treated group (**B**, **C** and **D**) and dl-NBP (10 mg/kg) treated group (**E**), respectively. Drugs were intravenously administered 10 min after the onset of MCAO. Infarcted tissue is white, whereas live tissue is darkly stained by TTC.

Fig. 3. Improvement in infarct volume of dl-PHPB in rats after 2 hours intraluminal suture occlusion of MCA and 24 hours reperfusion. Values are mean±S.E.M. (n=10). dl-PHPB (1.3, 3.9 and 12.9 mg/kg) and dl-NBP (dose displayed represent the actual doses of 10 mg/kg) were intravenously administered 10 min after the onset of MCAO. ** $P<$ 0.01, *** $P<0.001$ vs. Vehicle-control group.

Fig. 4. Relative rCBF measured with LDF in the MCA-supplied cortex of all groups over a period of 120 minutes after MCAO, then recordered for 50 minutes after reperfusion. Values are mean±S.E.M. (n=9). dl-PHPB (1.3, 3.9 and 12.9 mg/kg) was intravenously administered 10 min after the onset of MCAO. (**A**), rCBF over the entire period of measurement. (**B**), rCBF from 10 to 120 min after MCAO shown on an expanded scale. * $P < 0.05$, ** $P < 0.01$ vs. Vehicle-control group.

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Fig. 5. The conversion curve of dl-PHPB to dl-NBP in plasma. Values are mean±S.E.M. (n=3). The concentrations of dl-PHPB and dl-NBP were determined by HPLC method. after dl-PHPB was added into the plasma 2, 5, 10 and 20min, the plasma 100μl was deproteinated by 200ul methanol and they were centrifuged. The supernatant was used for analysis the drug level.

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Table 1. Physiological parameters in rats subjected to 2 hours intraluminal suture occlusion of MCA and 24 hours reperfusion.

Parameter	Vehicle -control	dl-PHPB			dl-NBP
		1.3 mg/kg	3.9 mg/kg	12.9 mg/kg	10 mg/kg
Before ischemia					
MABP (mm Hg)	99±6	100±5	104±6	98±7	91±12
Arterial pH	7.27±0.05	7.27±0.03	7.30±0.02	7.26±0.02	7.29±0.01
PaO_2 (mm Hg)	94.5±7.9	92.3±7.2	97.9±3.0	97.3±12.9	95.6±1.3
$PaCO_2$ (mm Hg)	53.9±4.8	52.1±3.9	51.1±0.9	50.7±8.9	53.7±0.3
Hematocrit (%)	48.1±1.4	48.5±1.0	47.3±2.3	48.8±0.6	49.2±0.6
Glucose (mg/dl)	167.6±10.5	166.9±9.4	167.2±14.4	174.3±15.0	170.7±11.4
Temperature (℃)	37.1±0.39	37.0±0.28	37.2±0.30	37.0±0.19	37.1±0.27
During ischemia					
MABP (mm Hg)	101±11	99±8	105±12	102±11	109±8
Arterial pH	7.24±0.03	7.23±0.04	7.27±0.09	7.23±0.04	7.25±0.02
PaO_2 (mm Hg)	101.5±12.2	103±5.8	99.8±8.5	100.8±6.4	97.4±2.8
$PaCO_2$ (mm Hg)	55.3±4.0	53.6±8.3	54.0±9.0	54.3±6.2	54.9±4.1
Hematocrit (%)	47.6±1.8	46.5±4.0	47.6±0.8	45.9±4.3	48.0±0.9
Glucose (mg/dl)	168.7±8.9	163.8±14.5	165.2±8.9	170.9±12.6	168.3±10.5
Temperature (℃)	36.8±0.34	36.7±0.21	36.8±0.28	36.7±0.42	36.9±0.31
After ischemia					
MABP (mm Hg)	98±9	103±6	100±7	96±12	93±9
Arterial pH	7.25±0.02	7.24±0.02	7.22±0.07	7.22±0.09	7.25±0.01
PaO_2 (mm Hg)	106.2±17.0	102.5±7.4	103.1±6.7	106.2±13.3	103.4±8.8
$PaCO_2$ (mm Hg)	56.1±3.7	52.1±11.3	57.7±8.7	54.8±4.4	56.6±4.9
Hematocrit (%)	48.0±1.4	49.1±3.4	47.3±4.7	48.8±0.6	49.2±0.6
Glucose (mg/dl)	170.1±11.9	169.2±15.1	168.6±9.5	178.1±11.8	169.2±9.8
Temperature (℃)	36.9±0.20	37.0±0.26	37.1±0.27	36.9±0.15	37.0±0.21
Temperature at Later reperfusion (℃)					
12h after MCAO	38.3±0.41	38.2±0.33	38.4±0.21	38.1±0.26	38.0±0.22
24h after MCAO	38.9±0.37	38.7±0.29	39.1±0.30	38.8±0.33	38.6±0.28

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Values are mean±S.D. (n=9). Physiological parameters were monitored at the time of 10 min before the onset of MCAO (Before ischemia), 30 min after the onset of MCAO (During ischemia), and 30 min after the end of MCAO (After ischemia). Besides the three time points above, body (rectal) temperature was still measured at 12 and 24 h after the onset of MCAO in some of animals (Later reperfusion, n=6). MABP, indicates mean arterial blood pressure; PaO_2, arterial oxygen tension; $PaCO_2$, arterial carbon dioxide tension.

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Table 2. Improvement in neurological behavior of dl-PHPB in rats after 2 hours intraluminal suture occlusion of MCA and 24 hours reperfusion.

Group	Neurological score
Vehicle-control	3.2±0.2##
dl-PHPB: 1.3 mg/kg	2.7±0.1*
3.9 mg/kg	2.1±0.2**
12.9 mg/kg	1.8±0.2**
dl-NBP: 10 mg/kg	2.0±0.2**
Sham-operate	0.0±0.0

Values are mean±S.E.M. (n=10). dl-PHPB and dl-NBP were intravenously administered 10 min after the onset of MCAO. ## $P<0.01$ vs. Sham-operate group, * $P<0.05$, ** $P<0.01$ vs. Vehicle-control group.

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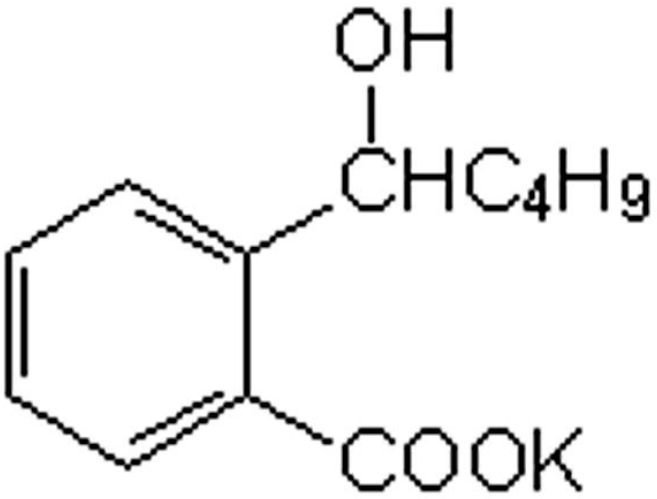
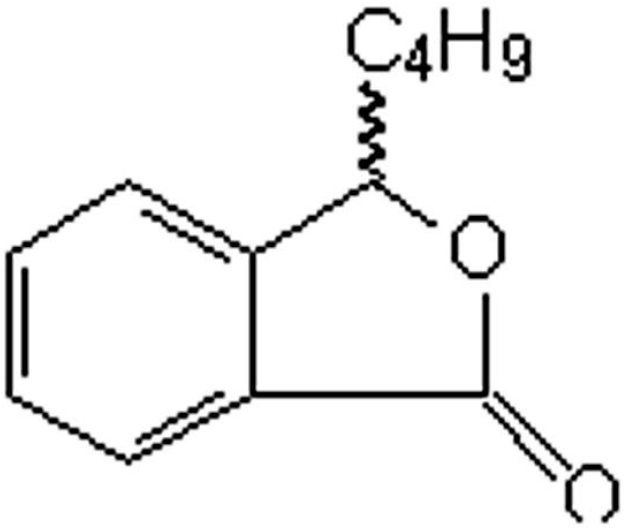
Figure 1



dl-PHPB

$C_{12}H_{15}O_3K$, FW: 246.4



dl-NBP

$C_{12}H_{14}O_2$, FW: 190.2

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Figure 2

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Figure 3

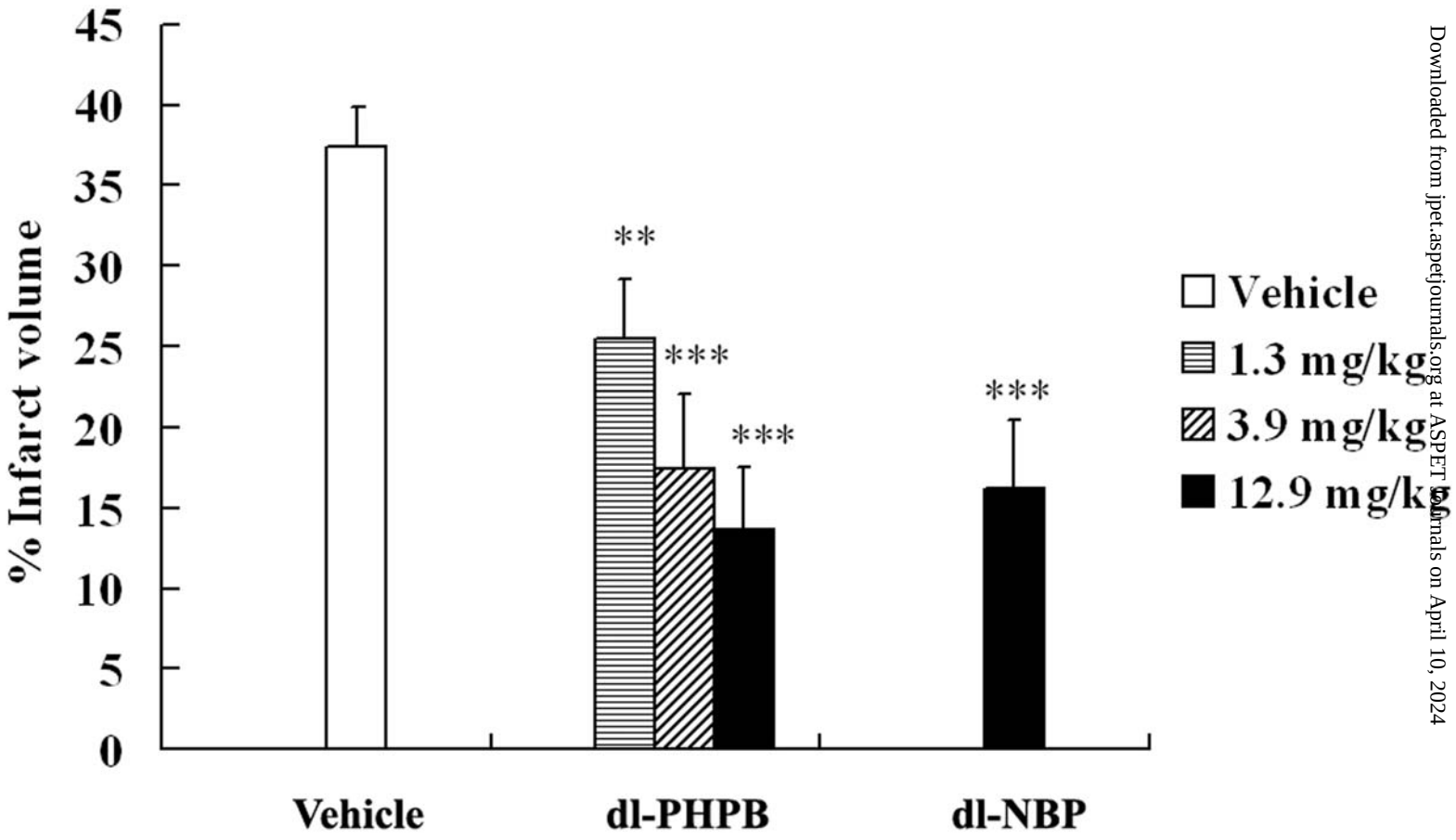

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Figure 4

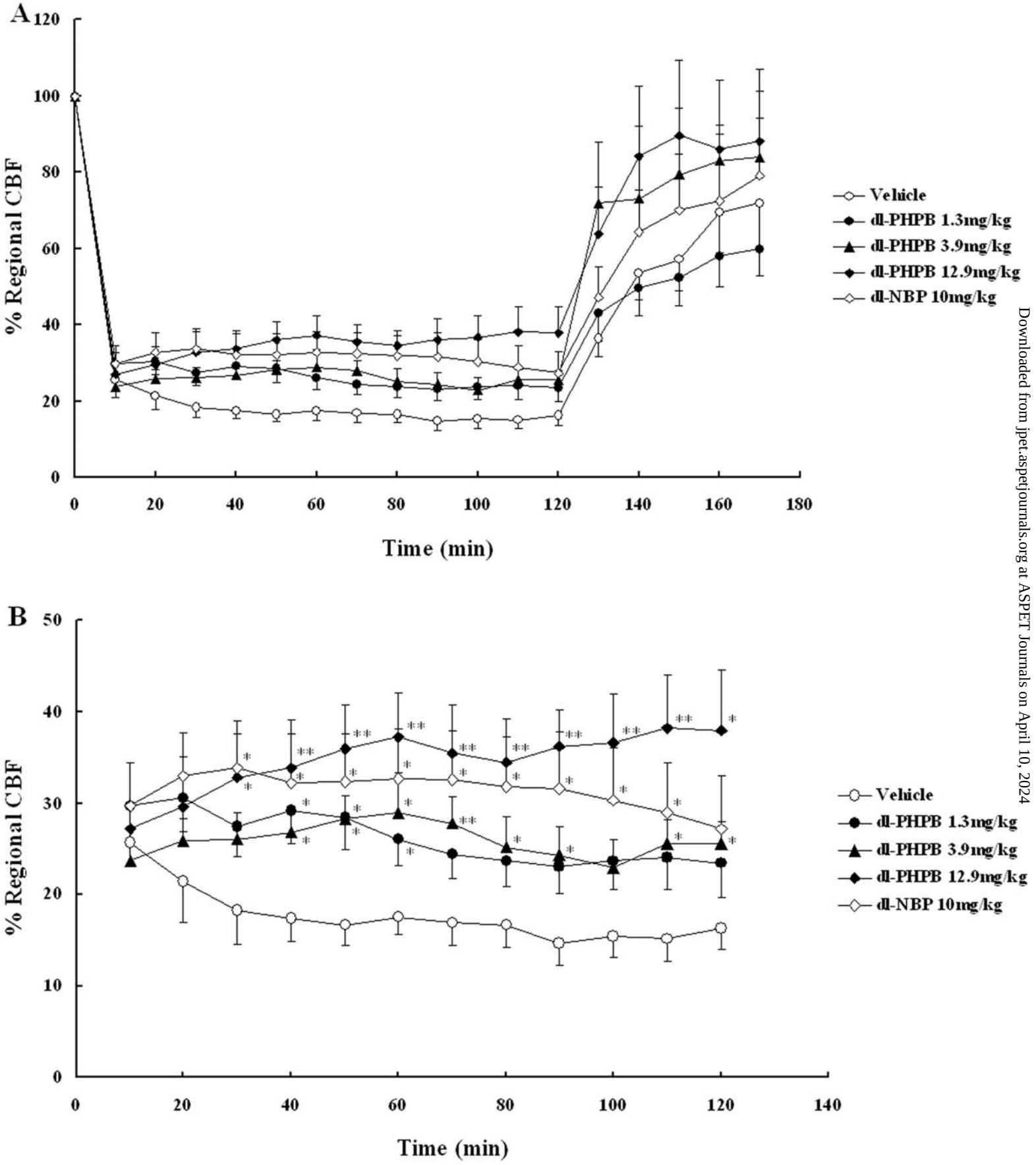

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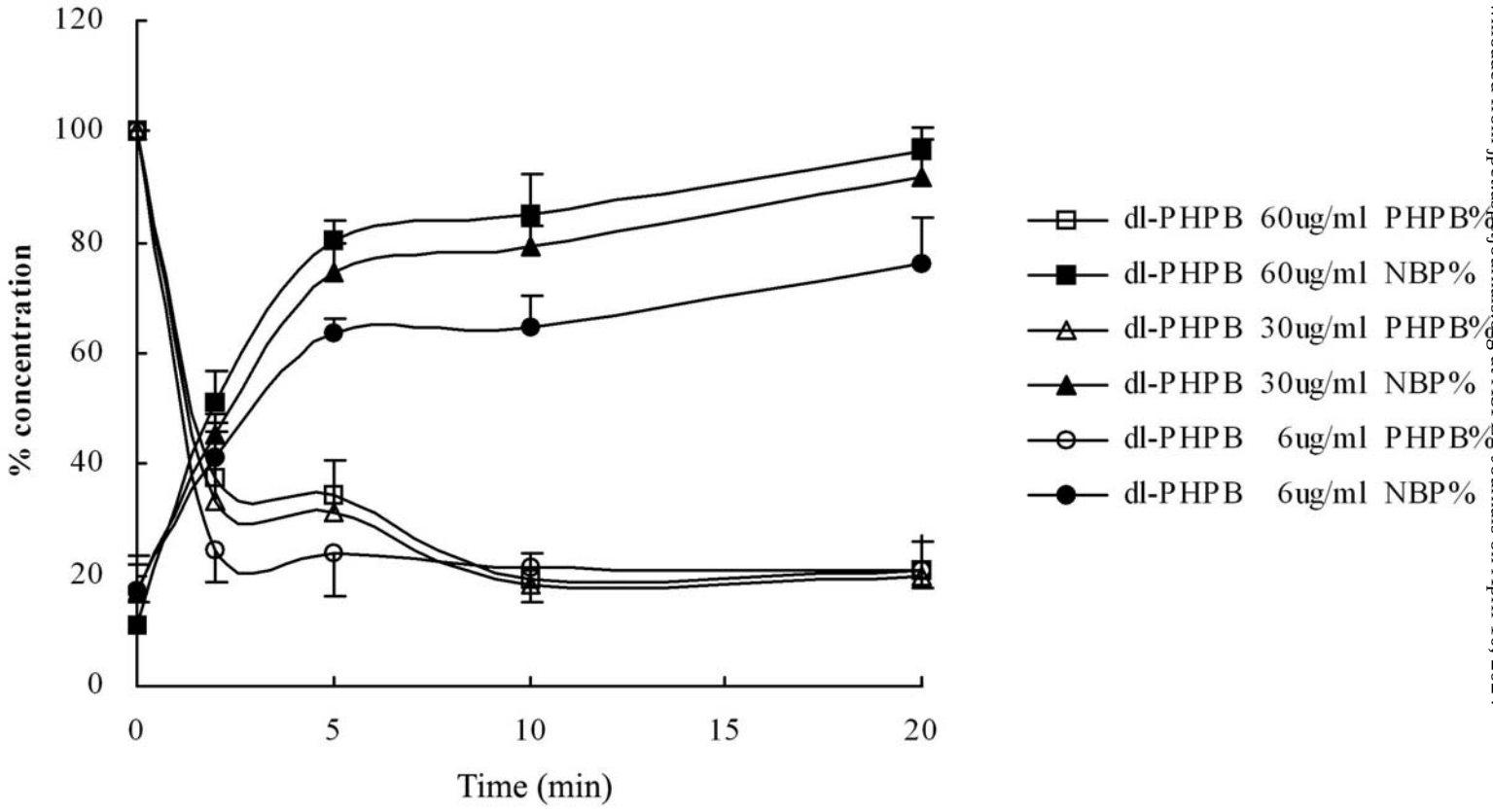
Figure 5



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