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RESEARCH ARTICLE

Protective Effects of 3-n-butylphthalide on Cerebral Infarction Induced by Local Ischemic Injury and Regulation Mechanism of the PI3K/Akt/GSK-3β Signaling Pathway

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Abstract: We aimed to study the protective effects of 3-n-butylphthalide (NBP) on cerebral infarction induced by local ischemic injury and regulation mechanism of the PI3K/Akt/GSK-3β signaling pathway. One hundred male Wistar rats aged 12-15 weeks were randomly divided into 5 groups (n=20). The middle cerebral artery occlusion (MCAO) model was established. NBP, P13K specific inhibitor LY294002 and NBP plus LY294002 groups were intraperitoneally administered on the first day after modeling, once a day for 7 days. Sham operation (Sham) and model groups were intraperitoneally given equal amounts of normal saline. Neuronal damage was detected by Nissl staining. Intact neurons were counted under light microscope. The protein expressions of Akt, P-Akt, GSK-3β and P-GSK-3β were detected by Western blotting. The mNS score of NBP group decreased significantly compared with that of model group (P<0.05). Compared with model group, the cerebral infarction volume of NBP group significantly reduced (P<0.05). Compared with model group, the number of intact neurons in NBP group significantly increased (P<0.05). Compared with model group, the phosphorylation levels of Akt and GSK-3β in NBP group significantly increased (P<0.05). By activating the PI3K/Akt/GSK-3β signaling pathway, NBP relieves neurological function damage and protects against cerebral infarction induced by local ischemic injury.

Keywords: *Butylphthalide, PI3K, Akt, GSK-3β, Apoptosis*

3-N-Butilftalidinin Lokal İskemik Hasarla İndüklenen Serebral Enfarktüs Üzerine Koruyucu Etkileri ve PI3K/Akt/GSK-3β Sinyal Yolunu Düzenleme Mekanizması

Öz: Bu çalışmada, 3-n-butilftalidinin (NBP) lokal iskemik hasarın neden olduğu serebral enfarktüs üzerine koruyucu etkileri ve PI3K/Akt/GSK-3β sinyal yolağını düzenlenme mekanizması incelenmiştir. 12-15 haftalık 100 erkek Wistar sıçan rastgele 5 gruba ayrıldı (n=20). Orta serebral arter oklüzyonu (MCAO) modeli oluşturuldu. NBP, P13K spesifik inhibitörü LY294002 ve NBP artı LY294002, modellemeden sonraki gün başlamak üzere ilgili gruplara 7 gün boyunca günde bir kez intraperitoneal olarak uygulandı. Sham ve model gruplarına intraperitoneal olarak eşit miktarda normal salin verildi. Nöronal hasar Nissl boyama ile tespit edildi. Sağlam nöronlar ışık mikroskobu altında sayıldı. Akt, P-Akt, GSK-3β ve P-GSK-3β protein ekspresyonları Western blotlama ile tespit edildi. NBP grubunun mNS skoru model grubuna kıyasla önemli ölçüde azalmıştı (P<0.05). Model grupla karşılaştırıldığında, NBP grubunun serebral enfarktüs hacmi önemli ölçüde azalmıştı (P<0.05). Model grupla karşılaştırıldığında, NBP grubundaki sağlam nöron sayısı önemli ölçüde artmıştı (P<0.05). Model grupla karşılaştırıldığında, NBP grubunda Akt ve GSK-3β fosforilasyon seviyeleri önemli ölçüde artmıştı (P<0.05). NBP, PI3K/Akt/GSK-3β sinyal yolunu aktive ederek nörolojik fonksiyon hasarını hafifletmekte ve lokal iskemik hasarın neden olduğu serebral enfarktüse karşı koruma sağlamaktadır.

Anahtar Sözcükler: *Butilftalid, PI3K, Akt, GSK-3β, Apoptozis*

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Introduction

Ischemic cerebral infarction is a clinically common central nervous system disease with high mortality and disability rates, which seriously threatens human health and quality of life [1]. With a complicated pathophysiological process, this disease is a blood flow disorder in the brain that is caused by various factors, leading to ischemia and hypoxia, apoptosis of neurons, functional damage of tissues and cells, as well as neurological dysfunction such as movement, language, sensation and memory [2]. At present, cerebral infarction is mostly treated by vascular recanalization in time, including thrombolysis and mechanical thrombectomy. Meanwhile, this therapy is usually combined with supportive treatments such as anticoagulation, reduction of intracranial pressure and nourishment of cranial nerves, which can relieve brain tissue damage and improve the prognosis. However, some cases still suffer from different degrees of hemiplegia, cognitive impairment, aphasia and swallowing dysfunction [3,4].

3-n-Butylphthalide (NBP) has significant therapeutic effects on the neurological function of patients with ischemic cerebral infarction [5]. It can enhance the blood circulation in the brain and prevent cerebral infarction caused by ischemia [6]. Meanwhile, NBP improves the energy metabolism in ischemic area and participates in the inhibition of neuronal apoptosis [7]. During cell apoptosis, the PI3K/Akt signaling pathway plays a key role in the regulation of related proteins, allowing membrane receptor signal to be transduced to cells for maintaining cell proliferation and inhibiting apoptosis [8]. As a substrate for Akt, GSK-3β is a key component regulating cell apoptosis [9]. The mechanism by which NBP protects against cerebral infarction induced by local ischemic injury by regulating the PI3K/Akt/GSK-3β signaling pathway remains unclear.

Therefore, we herein established a rat model of middle cerebral artery occlusion (MCAO) to assess the protective effects of NBP on cerebral infarction induced by local ischemic injury, and the regulation of the PI3K/Akt/GSK-3β signaling pathway. The findings provide valuable experimental evidence for clinical practice.

Material and Methods

Experimental Animals, Reagents and Apparatus

This study has been approved by the animal ethic committee of our hospital (approval No. TTH202001004), and great efforts have been made to minimize the animals' suffering. SPF-grade healthy Wistar male rats aged 12-15 weeks and weighing 200-220 g were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (China). They were kept in an animal breeding room with constant temperature and humidity, and adaptive feeding was carried out with a 12 h/12 h light/dark cycle. All experiments were in line with the 3R principle.

NBP was bought from Shijiazhuang Pharmaceutical Group NBP Pharmaceutical Co., Ltd. (China). LY294002 was obtained from Sigma (USA). BCA protein concentration detection kit, DAB chemiluminescence kit, and antibodies against Akt, P-Akt, GSK-3β and P-GSK-3β were provided by Shanghai Renjie Biotechnology Co., Ltd. (China). Magnetic resonance imaging (MRI) scanner was purchased from Siemens (Germany). Gel imaging system was bought from Bio-Rad (USA). BXM-950 optical microscope was obtained from Shanghai Bingyu Optical Instrument Co., Ltd. (China).

Model Establishment and Grouping

One hundred rats were numbered and then randomly divided into 5 groups (n=20): sham operation (Sham) group, model group, NBP (10 mg/kg) group, P13K specific inhibitor LY294002 (LY, 10 mg/kg) group and NBP (10 mg/kg) plus LY294002 (10 mg/kg) (NBP + LY) group. Model establishment: Under anesthesia, a nylon thread with 0.285 mm diameter was used to occlude blood flow in the anterior segment of MCA and its lateral branch for 2 h, causing local MCA ischemia without affecting blood flow in the anterior cerebral artery [10]. After the nylon thread was withdrawn and the MCA blood flow was restored, the rat MCAO model was successfully established. For the Sham group, only vascular ligation or occlusion was not performed. Each group was intraperitoneally injected with corresponding drugs on the first day after modeling, once a day for 7 days. The same amounts of normal saline were intraperitoneally given to the Sham and model groups.

Scoring of Modified Neurological Severity (mNS)

Thirty minutes after administration on the 7th day, the rat behaviors were evaluated by mNS scoring according to the criteria below. 0 point: Rat crawls normally without asymmetric movement; 1 point: forelimb or hind limbs are bent when the tail is lifted vertically; 2 points: rat fails to walk straight based on the criterion for 1 point; 3 points: rat circles leftward while crawling; 4 points: rat falls down leftward during free movement; 5 points: rat drags its left forepaw backward based on the criterion for of 4 points; 6 points: rat fails to support its body or to crawl by itself [11].

Measurement of Cerebral Infarction Volume by MRI

After mNS scoring, 6 rats were randomly selected. The cerebral infarction volume was detected by MRI scanner. The rats were placed in the supine position. After the standard axial position was found, the coronal plane was subjected to three-layer scan based on cross-sectional T2-weighted imaging, with a layer thickness of 1.5 mm and a spacing of 0.2 mm. T2-weighted imaging was carried out to measure the volume of cerebral infarction. The infarct

area was pale white, and the normal brain tissue area was gray. Cerebral infarction volume (%) = (infarct volume/ volume of whole brain tissue) x 100%. The calculation was conducted using ImageJ software [12].

Detection of Neurons in Brain Tissue by Nissl Staining

After mNS scoring, 6 rats were randomly selected. Under anesthesia, the chest and abdomen were incised, and the exposed left ventricle was intubated. 4% Paraformaldehyde was perfused until the heart turned white. Then the brain tissue was taken out, placed on ice, immediately fixed with 4% paraformaldehyde, dehydrated under vacuum conditions with gradient concentrations of ethanol solutions, transparentized with xylene and paraffin-embedded. The treated brain tissue was thereafter sliced into 5 μm-thick coronal sections that were subjected to Nissl staining. Six different visual fields were observed under an optical microscope at the magnification of 400x, and images were processed by Image-Pro 6.2 software to count intact neurons in the cerebral cortex on the ischemic side [13].

Detection of Akt, P-Akt, GSK- 3β and P-GSK-3β Protein Expressions by Western Blotting

After mNS scoring, 6 rats were randomly selected. Under anesthesia, they were killed by cervical dislocation, from which the skull was rapidly disconnected to collect brain tissue on the ischemic side. Subsequently, the cerebral cortex was separated in an ice bath and stored in liquid nitrogen. The brain tissue was homogenized with RIPA lysis buffer, left still in ice bath for 5 min and centrifuged at 13000 rpm and 4°C for 10 min to collect the supernatant. Protein concentration in the supernatant was measured by BCA protein quantification kit. Afterwards, protein samples were resolved by 10% SDS-PAGE, and the product was electronically transferred onto a PVDF membrane. Then the membrane was blocked by 5% TBST at room temperature, incubated overnight with primary antibodies (1:1000 diluted) at 4°C, washed by TBST for 10 min, incubated with HRP-labeled goat anti-rabbit IgG secondary antibody (1:10000 diluted) at room temperature for 2 h, developed by DAB solution and observed by gel imaging analyzer. The relative expression levels of target proteins were detected by using GAPDH as the internal reference.

Statistical Analysis

All data were analyzed by SPSS16.0 software. The categorical data conforming to normal distribution were expressed as mean ± standard deviation. Multigroup comparisons were performed by one-way analysis of variance, and pairwise comparisons were conducted with the independent t test. P<0.05 was considered statistically significant.

Results

Compared with the Sham group, the mNS score of the model group was significantly higher (P<0.05). After NBP treatment, the mNS score of the NBP group decreased significantly compared with that of the model group (P<0.05), but the scores of LY and NBP + LY groups were similar to that of the model group (P>0.05) *(Fig. 1)*, indicating that LY294002 blocked NBP from improving the neurological function of MCAO rats.

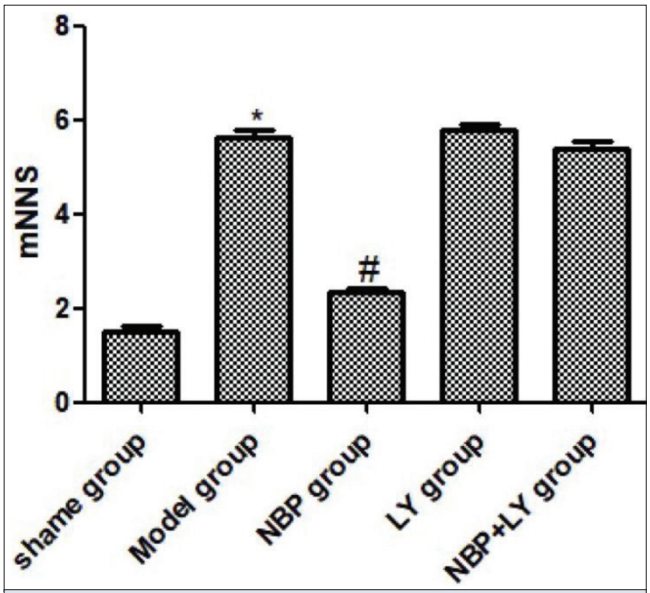

Fig 1. Effects of NBP on neurological function of MCAO rats. *Compared with Sham group, P<0.05; #compared with model group, P<0.05

No infarct area was found in the brain tissue of the Sham group. Compared with the Sham group, the cerebral infarction volume of the model group significantly increased (P<0.05). Compared with the model group, the cerebral infarction volume of the NBP group significantly reduced (P<0.05), whereas the volumes of LY and NBP + LY groups were not significantly different (P>0.05) *(Fig. 2)*, suggesting that LY294002 inhibited NBP from repairing ischemic cerebral injury.

The Sham group had normal neuron morphology. Compared with the Sham group, the model group had abnormal structures of neurons and significantly decreased number of intact neurons (P<0.05). Compared with the model group, the number of intact neurons in the brain tissue of the NBP group significantly increased (P<0.05), but the numbers of LY and NBP + LY groups were not significantly different (P>0.05) *(Fig. 3)*, indicating that LY294002 suppressed NBP from protecting the neurons undergoing ischemic brain injury.

Western blotting showed that compared with the Sham group, the phosphorylation levels of Akt and GSK-3β in the

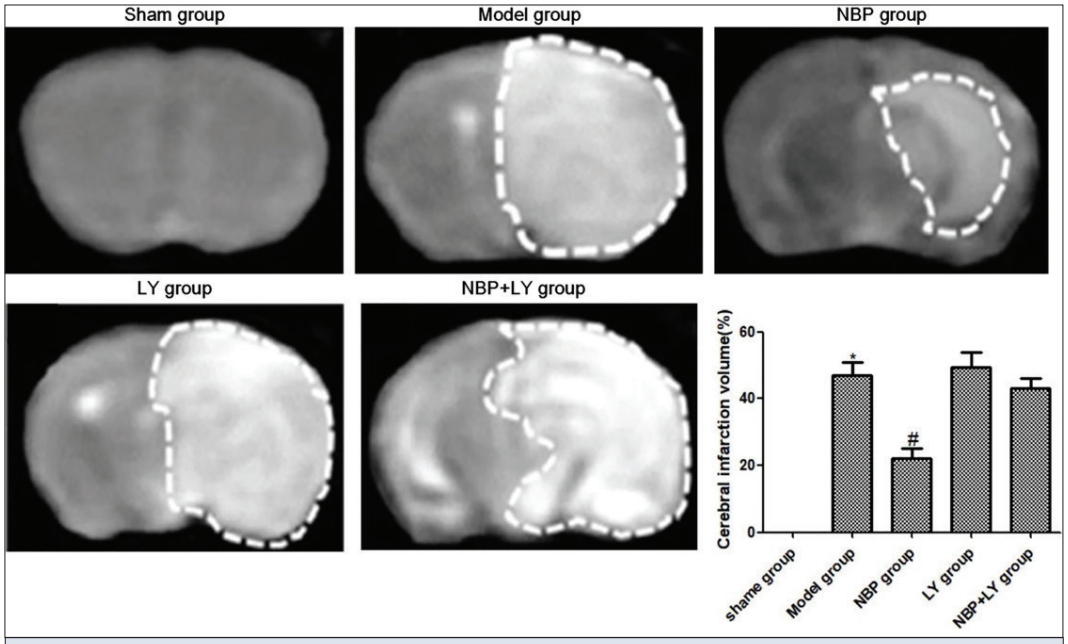

Fig 2. Effects of NBP on cerebral infarction volume of MCAO rats. *Compared with Sham group, P<0.05; #compared with model group, P<0.05

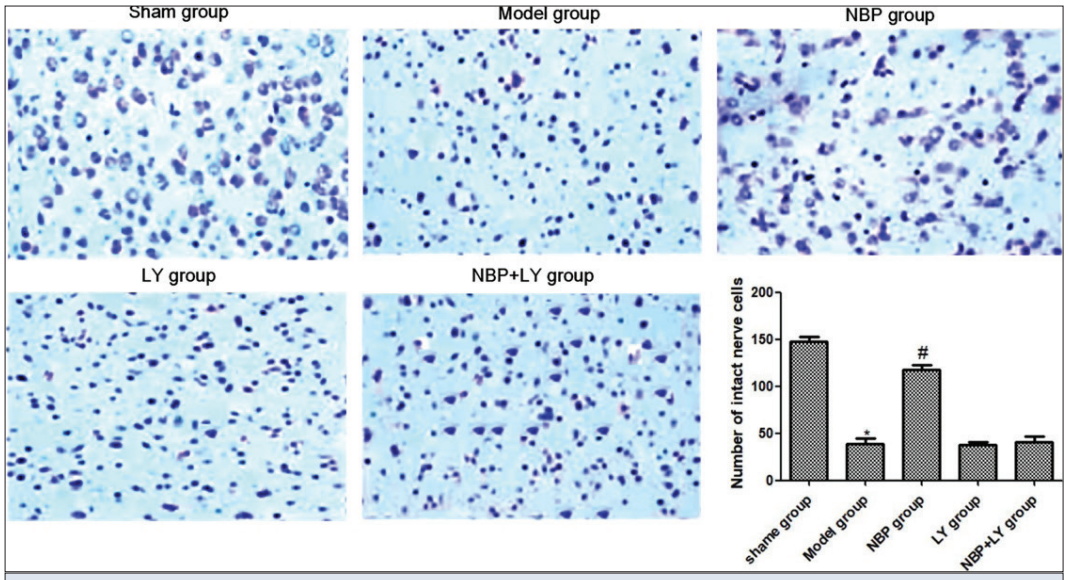

Fig 3. Nissl staining results for effects of NBP on cerebral neuronal integrity of MCAO rats (number of neurons in a ×400 visual field). *Compared with Sham group, P<0.05; #compared with model group, P<0.05

model group significantly decreased (P<0.05). Compared with the model group, such levels in the brain tissue of the NBP group significantly increased (P<0.05), whereas the levels of LY and NBP + LY groups were not significantly different (P>0.05) *(Fig. 4)*, revealing that LY294002 counteracted the therapeutic effects of NBP by decreasing the levels of in brain tissues with ischemic injury.

Discussion

The impairment of brain function has serious adverse effects on physiological activities [14]. Particularly, ischemic cerebral infarction poses a great threat to human life [15]. NBP has been widely used as a therapeutic drug for ischemic cerebrovascular disease in clinical practice, with remarkable effects [16]. In this study, a MCAO model was first established, and then the concentration of butylphthalide was selected based on preliminary experiments. Compared with the model group, NBP-treated rats had significantly reduced volume of cerebral infarction, alleviated neurological damage and decreased number of apoptotic neurons, verifying that cerebral ischemic injury was alleviated. The results are consistent

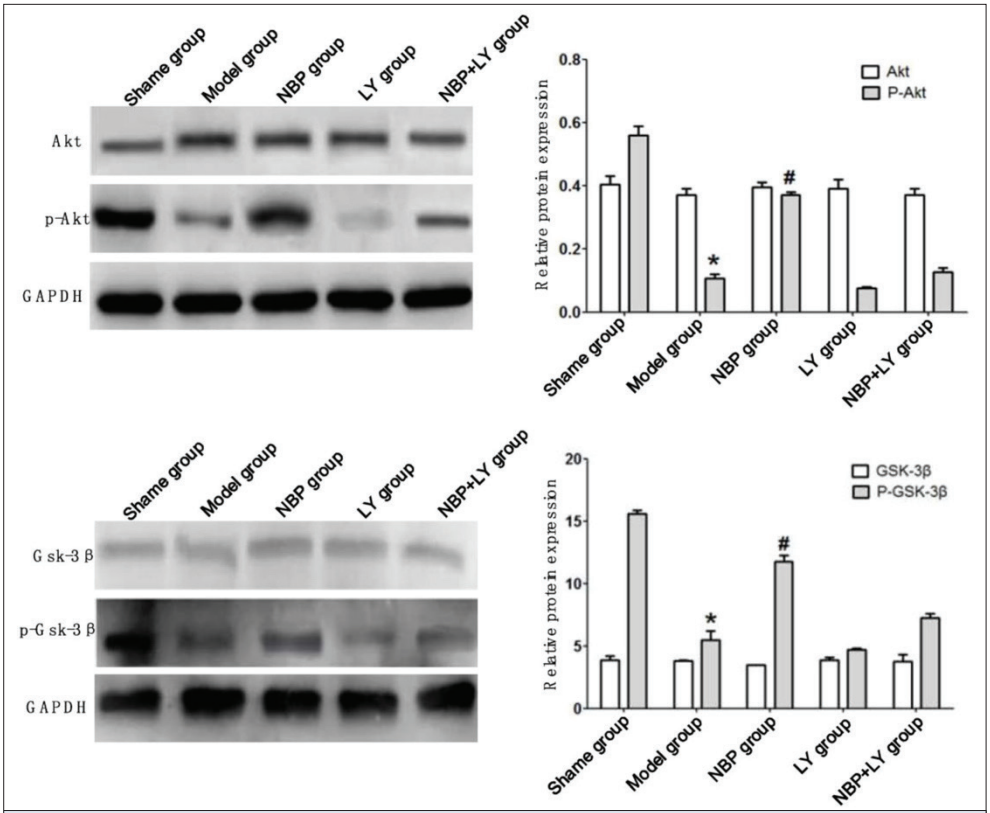

Fig 4. Effects of NBP on expressions of Akt, P-Akt, GSK-3β and P-GSK-3β in brain tissues of MCAO rats. *Compared with Sham group, P<0.05; #compared with model group, P<0.05

with a previous literature [17], confirming the therapeutic effects of NBP again.

The PI3K/Akt signaling pathway plays crucial roles in cell migration, mobilization, differentiation and apoptosis resistance [18]. As a key target protein, PI3K promotes the phosphorylation of GSK-3β in downstream pathways by phosphorylating Akt through PDK1 [19]. Subsequently, activated GSK-3β inhibits cellular oxidative stress and inflammatory response, and also predominantly resists apoptosis [20]. Many kinds of herbal and insect medicine have been reported to mitigate ischemic stroke-induced damage via the PI3K/Akt signaling pathway [21]. By enhancing the activity of mitochondrial ATPase, NBP protects mitochondria from ischemic damage, thereby suppressing cell apoptosis [22]. In addition, NBP may increase the expressions of VEGF and bFGF by acting on the ischemic site of the brain to protect against damage [23]. However, the mechanism remains largely unknown.

As a PI3K inhibitor, LY294002 specifically inhibits the activity of PI3K110 subunit and blocks PI3K-mediated signaling pathway [24]. In this study, LY294002 was used to clarify the mechanism by which NBP regulated the PI3K/Akt/GSK3β signaling pathway to protect against local brain injury. The phosphorylation levels of Akt and GSK-3β were significantly augmented in the NBP group compared with those of the model group, and the levels of the LY+ NBP group decreased significantly compared with those of the NBP group. Accordingly, LY294002 reversed NBP-mediated phosphorylation of Akt and GSK3β, indicating that NBP allowed Akt and GSK3β phosphorylation through PI3K [25].

It has previously been reported that the PI3K/Akt/GSK-3β signaling pathway inhibited cell apoptosis after reperfusion injury in the brain, and then participated in the repair process, playing a protective role [26]. Likewise, we herein found that compared with the NBP group, the cerebral infarction volume of the LY + NBP group was significantly enlarged, neurological damage was aggravated, and the number of apoptotic neurons was increased. Hence, NBP may protect against cerebral ischemic injury by regulating the PI3K/Akt/GSK-3β signaling pathway.

In summary, by activating the PI3K/Akt/GSK-3β signaling pathway, NBP relieves neurological function damage and protects against cerebral infarction induced by local ischemic injury. Nevertheless, whether other pathways are involved still needs further in-depth studies.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author (J. Zhang) on reasonable request.

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This study was not financially supported.

Conflict of Interest

There is no conflict of interest.

Authors' Contributions

KL designed this study, prepared this manuscript, analyzed experimental data; YC analyzed experimental data, manuscript writing.

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