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**Effect of dl-3-n-butylphthalide on infarction volume in animal models of ischemic stroke: A meta-analysis**

Luan D *et al.* Dl-3-n-butylphthalide reduces cerebral infarction volume

Di Luan, Zheng-Yu Wu, Yuan-Xiang Zhang, Li-Li Yuan, Yang Xu, Zhao-Hu Chu, Ling-Song Ma, Ya-Ping Wang, Shou-Cai Zhao

**Di Luan, Li-Li Yuan, Yang Xu, Zhao-Hu Chu, Ling-Song Ma, Ya-Ping Wang, Shou-Cai Zhao,** Department of Neurology, Yijishan Hospital Affiliated to Wannan Medical College, Wuhu 241000, Anhui Province, China

**Zheng-Yu Wu,** **Yuan-Xiang Zhang,** Department of Clinical Pharmacy, Yijishan Hospital Affiliated to Wannan Medical College, Wuhu 241000, Anhui Province, China

**ORCID number**: Di Luan (0000-0002-8832-7555); Zheng-Yu Wu (0000-0002-0416-0760); Yuan-Xiang Zhang (0000-0002-0317-9435); Li-Li Yuan (0000-0002-5497-6338); Yang Xu (0000-0001-8778-3885); Zhao-Hu Chu (0000-0002-8103-5533); Ling-Song Ma (0000-0001-9035-0159); Ya-Ping Wang (0000-0002-1753-0863); Shou-Cai Zhao (0000-0001-8555-4835).

**Author contributions**: Luan D, Wu ZY, Zhang YX, Yuan LL, and Zhao SC conceived and designed the study; Luan D and Wu ZY performed the study search, study screening and selection, and data extraction; Wang YP performed the analysis and supervised the study; all authors were involved in interpretation of the results; Luan D finalized the manuscript; all authors read and approved the final manuscript.

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**Corresponding author: Shou-Cai Zhao, MD, Doctor, Professor, Teacher,** Department of Neurology, Yijishan Hospital affiliated to Wannan Medical College, Wuhu 241000, Anhui Province, China. [neurozsc@wnmc.edu.cn](mailto:neurozsc@wnmc.edu.cn)

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**Abstract**

***BACKGROUND***

Ischemic stroke is a frequently-occurring disease in the elderly and characterized by high morbidity and mortality. Dl-3-n-butylphthalide (NBP), a synthetic compound based on natural celery seeds, has potential therapeutic effects on cerebral ischemia, brain trauma, memory impairment, and epilepsy.

***AIM***

To evaluate the effect of NBP on infarct volume in experimental ischemic stroke.

***METHODS***

Twenty-one relevant articles were included from the PubMed, EMBASE, Web of Science, Chinese National Knowledge Infrastructure, VIP information database, and Wanfang database, and data on the effect of dl-3-n-butylphthalide on infarction volume in the middle cerebral artery occlusion models were extracted. Statistical analyses of standard mean difference (SMD) were performed using a random effects model with Revman 5.3.

***RESULTS***

Meta-analysis of the 21 studies suggested that NBP significantly reduced the cerebral infarction volume in middle cerebral artery occlusion model animals compared to the control group [SMD = -3.97, 95%CI: -4.71 to -3.23, *P* < 0.01; heterogeneity: χ2 = 59.09, df = 20 (*P* < 0.01); *I*2 = 66 %].

***CONCLUSION***

NBP is effective in the treatment of experimental ischemic stroke.

**Key words:** Butylphthalide; Animal model; Ischemic stroke; Meta-analysis

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**Core tip:** The systematic review of animal research is of great significance in drug development. This study reports for the first time a systematic review and meta-analysis of the effects of butylphthalide on the volume of cerebral infarction in experimental ischemic stroke.

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**INTRODUCTION**

Ischemic stroke is a frequently-occurring disease in the elderly and characterized by high morbidity and mortality[1,2]. The current treatment includes drug-based thrombolysis and interventional therapy in acute stage, however, there are many inherent limitations in them[3,4]. To date, more than 1000 clinical trials of potential neuroprotective drugs have been verified to fail[5].

Dl-3-n-butylphthalide (NBP), a synthetic compound based on natural celery seeds, has potential therapeutic effects on cerebral ischemia, brain trauma, memory impairment, and epilepsy; the injectable formulations have been approved for the treatment of acute ischemic stroke in China[6]. NBP protects the integrity of cerebrovascular structures[7], promotes the formation of collateral circulation, accelerates the proliferation of neonatal capillary[8,9], and increases the cerebral blood perfusion[10]; by targeting mitochondria, it improves neuronal energy metabolism[11] and reduces oxidative stress damage and neuronal apoptosis[12]. The systematic review of animal research is of great significance in drug development[13]. To this end, we conducted a meta-analysis of preclinical studies to evaluate the efficacy and mechanisms of NBP in experimental ischemic stroke.

**MATERIALS AND METHODS**

***Literature search strategies***

This meta-analysis was based on the Preferred Reporting Items for Systematic Reviews and Meta-Analyses statement[14]. All Chinese and English articles published before August 2018 regarding the effects of NBP on experimental ischemic stroke were searched in the six databases, which included PubMed, EMBASE, Web of Science, Wanfang database, VIP Chinese Journal Service Platform database, and China National Knowledge Infrastructure database. Furthermore, to further retrieve the relevant literature, we searched the list of references for potential publications. In the search of the Web of Science, PubMed, and EMBASE databases, only one keyword of “butylphthalide” was used. In the search of other databases, the following search strategy was utilized: “butylphthalide” AND “cerebral ischemia OR brain ischemia OR cerebral infarction OR brain infarction OR stroke OR cerebral ischemia/reperfusion OR cerebral I/R”.

***Inclusion and exclusion criteria***

The inclusion criteria were: (1) the experimental ischemic stroke model was established by the adoption of middle cerebral artery occlusion (MCAO); (2) the intervention group used NBP and the control group applied blank or non-functional solvent; and (3) the cerebral infarct volume was included in the study results, the unit of infarct volume was “%”, and the calculation formula was (infarction volume / whole brain volume) × 100%. The exclusion criteria were: (1) the intervention group was not administered with NBP or given NBP with other medicines concomitantly; (2) MCAO was not adopted for developing the animal models; (3) no control group was included; (4) repeating literature; and (5) the data were not available.

***Literature screening and data extraction***

By reading the title, abstract, and full text according to the inclusion and exclusion criteria, the literature and extracted data were screened independently and cross-checked by the first author and the second author. When there was a disagreement, the point must be reached through the discussion panel which consisted of all authors, and the final conclusion was determined by the corresponding author.

The following data were extracted from the included literature: (1) the year of publication and the name of the first author; (2) the species, age, weight, gender, anesthesia methods, and model types of experimental animals (transient MCAO or permanent MCAO); (3) therapeutic dose, route of administration, time of onset of treatment, and duration of interventional treatment; (4) the mean value and standard deviation of the cerebral infarction volume; and (5) the potential therapeutic mechanism of NBP for ischemic stroke.

Moreover, in case of the use of multiple doses and multiple time points across studies, the final experimental data using the highest dose were extracted. If the volume of cerebral infarction cannot be obtained directly from the original text, the author of the literature was contacted by e-mail to get complete data, and if not, calculation was performed by using digital scale software.

***Quality assessment***

The risk of bias tool of the Systematic Review Centre for Laboratory animal Experimentation’s was applied to assess the methodological quality of the included studies[15]: (1) baseline characteristics: the strain, gender, age, weight, anesthesia methods, name, and anesthetic dose of the experimental animals were involved; (2) allocation concealment: the experimental animals were grouped randomly; (3) sequence generation: the generation of allocation sequence was random; (4) random housing: the living environment and feeding conditions of each group of animals were in conformity with those of each group; (5) blindly feeding: the blind method was adopted for the breeder; (6) random outcome assessment: the outcomes were evaluated randomly on the premise of random selection of animals; (7) result evaluator blindness: the blind method was adopted by the outcome evaluator; (8) complete outcome data: all data of animals were included in the final analysis; (9) no selective outcome reporting: no report bias; and (10) no other sources of bias: there were no other factors that contributed to the risk of high bias.

***Statistical analysis***

Revman 5.3 software was used to analyze all data. Since the cerebral infarction volume was considered as continuous data, the standard mean difference was used to assess the combined effect sizes using a random effects model.

**RESULTS**

***Study inclusion***

A total of 5149 relevant articles were retrieved from six databases, among which 4630 duplicate or irrelevant articles were excluded, resulting in 519 articles for further evaluation. After scanning the titles and abstracts of the remaining articles, 449 were rejected for being review comments, reviews, case reports, clinical trials, and editorials. By reading the full text of the remaining 70 articles, 49 were eliminated as the animal model was not established by the MCAO method, the intervention drug was not NBP monotherapy, there was no control group, or the infarct volume calculation formula did not meet the inclusion criteria. Ultimately, 21 eligible articles were identified[8,16-35] (Figure 1).

***Study characteristics***

A total of 21 articles were collected, among which 5[24,25,32,33,35] were published in Chinese and the remaining were in English. A total of 314 animals, including 159 in the experimental group and 155 in the control group, were involved in 21 studies to investigate the effect of NBP on the volume of cerebral infarction in the experimental ischemic stroke model. Moreover, in 21 articles, there were 15 studies performed on SD rats (15/21, 71.4%), 3[25,29,35] on Wistar rats (3/21, 14.3%), 1[22] on C57BL6/J mice (1/21, 4.8%), 1[16] on CD1 mice (1/21, 4.8%), and 1[31] on 129S2/Sv mice (1/21, 4.7%); 21 studies used male animals (20/21, 95.2%), and one used both male and female animals (1/21, 4.8%)[18]. As for anesthesia method, intraperitoneal injection of chloral hydrate was used in 14 (14/21, 66.7%) studies, injection of sodium pentobarbital into the abdominal cavity in 1(1/21, 4.8%)[19], ketamine and xylazine injection into the peritoneal cavity in 1(1/21, 4.7%)[29], and isoflurane inhalation in 1(1/21, 4.8%)[22]. Only one study[34] adopted both atropine sulfate to reduce airway secretions and inhalation anesthesia with isoflurane (1/21, 4.7%). In addition, 3 studies[17,18,33] did not mention the anesthesia methods (3/21, 14.3%). The animal models used in 15 studies were transient MCAO (tMCAO) (15/21, 71.4%), and a permanent MCAO (pMCAO) was adopted in 6 (6/21, 28.6%) studies[16,25,29,33,35]. To detect infarction volume, 19 and 2[8,22] studies used 2,3,5-triphenyltetrazolium chloride and cresyl violet as the staining agents, respectively (Table 1).

***Study quality***

Of the 21 studies, five got 7 points, four got 6, eight got 5, two got 4, and two got 3. None of the studies described blind feeding and random outcome assessment; the result evaluator blindness was described only in 2 studies[8,32]; all studies described the data of baseline characteristics; 2 studies[18,29] found other sources of bias; no incomplete outcome data, and no selective outcome reporting were described in 11 and 17 studies, respectively (Table 2).

***Effectiveness***

Meta-analysis of the 21 studies suggested that NBP significantly reduced the cerebral infarction volume of MCAO model animals compared to the control group [SMD = -3.97, 95%CI: -4.71 to -3.23, *P* < 0.01; heterogeneity: *χ*2 = 59.09, df = 20 (*P* < 0.01); *I*2 = 66 %] (Figure 2). Moreover, meta-analysis of 15 studies adopting the tMCAO model also verified that NBP significantly reduced infarct volume [SMD = -3.67, 95%CI: -4.52 to -2.82, *P* < 0.01; heterogeneity: χ2 = 42.34, df = 14 (*P* < 0.01); *I*2 = 67%] (Figure 3A). The same is true for studies using the pMCAO model [SMD = -4.70, 95%CI: -5.92 to -3.47, *P* < 0.01; heterogeneity: χ2 = 9.26, df = 5 (*P* = 0.10); *I*2 = 46%] (Figure 3B). When analyzing the effects of pre- or post-administrated NBP on the volume of cerebral infarction in the MCAO model, it was found that both pre-administration [SMD = -3.93, 95%CI: -5.51 to -2.36, *P* < 0.01; heterogeneity: χ2 = 25.58, df= 6 (*P* < 0.01); *I*2 = 77%] (Figure 4A) and post-administration [SMD = -3.62, 95%CI: -4.32 to -2.92, *P* < 0.01; heterogeneity: χ2 = 17.92, df = 11 (*P* =0.08); *I*2 = 39%] (Figure 4B) reduced the infarct volume of the model animals. A funnel plot was adopted to evaluate publication bias and slight bias was found (Figure 5A).

**DISCUSSION**

***Summary of evidence***

The preclinical meta-analysis study evaluated the effects of NBP on infarct volume in experimental ischemic stroke, which was based on experimental data from 314 animals in five Chinese articles and sixteen English articles. The evidence obtained from the present study suggests that NBP might play potential neuroprotective roles for ischemic stroke by increasing cerebral blood flow, enhancing mitochondrial function, protecting integrity of the structure and function of blood-brain barrier, and exerting anti-inflammatory and antioxidant stress activities.

***Limitations***

First, the absence of the relevant literature in other languages other than Chinese and English may lead to selective bias. Second, none of studies provided sample size calculations, blind feeding, and random outcome assessment. Third, the lack of negative research may result in an overestimation of the efficacy of NBP. Fourth, cerebral infarction is usually accompanied by other conditions, such as old age, hypertension, hyperlipidemia, diabetes, and heart disease[36-39]. We did not analyze the effects of NBP on cerebral infarction when the accompanying situation occurred. Fifth, one study[18] using female animals did not rule out estrogen neuroprotection, which has been reported[40]. One study[29] of anesthetic drugs containing ketamine did not eliminate its neuroprotection, which has been reported in preclinical and clinical studies[41,42].

***Potential neuroprotective mechanisms***

Summarizing the included literature, we found that NBP plays a neuroprotective role in experimental ischemic stroke by acting on multiple targets (Figure 5B). We drew a conclusion of the underlying mechanisms as follows: (1) Increasing blood supply to brain tissue in the ischemic area by dilating the middle cerebral artery[8], regulating the expression of *REN, AGT, ACE 1, AGTR 1, RoA, PTGIS, PTGES,* and *TBXAS 1* in ischemic brain tissue[8], decreasing TXB2 and 6-keto-PGF1α ratio[27], and reducing thrombosis; (2) Promoting angiogenesis by increasing VEGF and bFGF in ischemic brain tissue[16,26,32,33]; (3) Exerting anti-inflammatory activity by inhibiting the TLR4/NF-κB signaling pathway[19,21] and decreasing the expression of S100B in ischemic brain tissue[25]; (4) Protecting the structure and function of the blood-brain barrier by modulating the expression of *MMP-9* and *claudin-5* in ischemic brain tissue[16,22], upregulating the expression of *GFAP* in ischemic brain tissue, stabilizing astrocytes[16], increasing the expression of TIMP1, and decreasing the expression of SBP in ischemic brain tissue[22]; (5) Exerting antioxidative stress activity by enhancing the Nrf-2/HO-1 signaling pathway[16], reducing the expression of ROS and MDA in ischemic brain tissue, and increasing the expression of SOD, GSH-px, GSH, Trx, and Txnip in ischemic brain tissue[17,20,21,28]; (6) Protecting the structure and function of mitochondria by increasing the expression of Bcl-2 in ischemic brain tissue[21], reducing the expression of Smac in ischemic brain tissue[25], and reducing mitochondrial release of cytochrome C and AIF[31]; and (7) Exerting anti-apoptosis activity by reducing the expression of cleaved caspase-3, p-p38, and p-JNK in ischemic brain tissue[17,31], increasing the expression of HGF and p-ERK in ischemic brain tissue[19,22], decreasing the expression of GRP78 and CHOP in ischemic brain tissue, and inhibiting endoplasmic reticulum stress-induced apoptosis[24].

***Implications***

Animal experiments are an important link between basic research and clinical experiments. The results have reference value for the next step in designing and implementing clinical research. Compared with clinical research, the principles of randomization and blindness are theoretically easier to be implemented in animal experiments. Animal research is important for comprehending disease mechanisms, and high-quality preclinical research is also critical for translational medicine[43,44]. Therefore, to obtain more accurate and less biased experimental data, designing animal programs should follow the guidelines all the time[15,45], calculate sample size in the beginning, apply applicable animals, use appropriate anesthetic drugs, adopt random feeding, blind models during the experiment, and employ random outcome measurements at the time of evaluation.

Similar to artemisinin, NBP is also a plant-derived drug approved for the treatment of acute ischemic stroke in China. We envision that NBP may be used to treat more patients in the world, like artemisinin, and it requires a large number of randomized, double-blind, and multi-center clinical trials in terms of safety and efficacy.

In conclusion**,** we have conducted the first preclinical systematic review and meta-analysis of the effects of NBP on experimental ischemic stroke, and found that NBP is effective in the treatment of experimental ischemic stroke.

**ARTICLE HIGHLIGHTS**

***Research background***

Ischemic stroke is a frequently-occurring disease in the elderly and characterized by high morbidity and mortality. Dl-3-n-butylphthalide (NBP), a synthetic compound based on natural celery seeds, has potential therapeutic effects on cerebral ischemia, brain trauma, memory impairment, and epilepsy. The systematic review of animal research is of great significance in drug development.

***Research motivation***

There are many studies on the therapeutic effects of NBP in the middle cerebral artery occlusion model, and there is controversy about whether NBP reduces the volume of cerebral infarction.

***Research objectives***

To evaluate the effect of NBP on infarct volume in experimental ischemic stroke.

***Research methods***

We searched Chinese and English databases to screen NBP-related literature. Data such as cerebral infarction volume and potential therapeutic mechanisms were extracted. The risk of bias tool of the Systematic Review Centre for Laboratory animal Experimentation’s was applied to assess the methodological quality of the included studies. Data analysis was performed using Revman 5.3 software.

***Research results***

Meta-analysis of the 21 studies had suggested that NBP significantly reduced the cerebral infarction volume of middle cerebral artery occlusion (MCAO) model animals compared to the control group. Moreover, meta-analysis of 15 studies adopting the tMCAO model also verified that NBP reduced infarct volume significantly. The same is true for studies using the pMCAO model. When analyzing the effects of pre- or post-administrated NBP on the volume of cerebral infarction in the MCAO model, it was found that both pre-administration and the post-administration reduced the infarct volume of the model animals.

***Research conclusions***

NBP is effective in the treatment of experimental ischemic stroke.

***Research perspectives***

Animal experiments are an important link between basic research and clinical experiments. The results have reference value for the next step in designing and implementing clinical research. Compared with clinical research, the principles of randomization and blindness are theoretically easier to be implemented in animal experiments. Animal research is important for comprehending disease mechanisms, and high-quality preclinical research is also critical for translational medicine. Therefore, to obtain more accurate and less biased experimental data, designing animal programs should follow the guidelines all the time, calculate sample size in the beginning, apply applicable animals, use appropriate anesthetic drugs, adopt random feeding, blind models during the experiment, and employ random outcome measurements at the time of evaluation.

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**Specialty type:** Medicine, research and experimental

**Country of origin:** China

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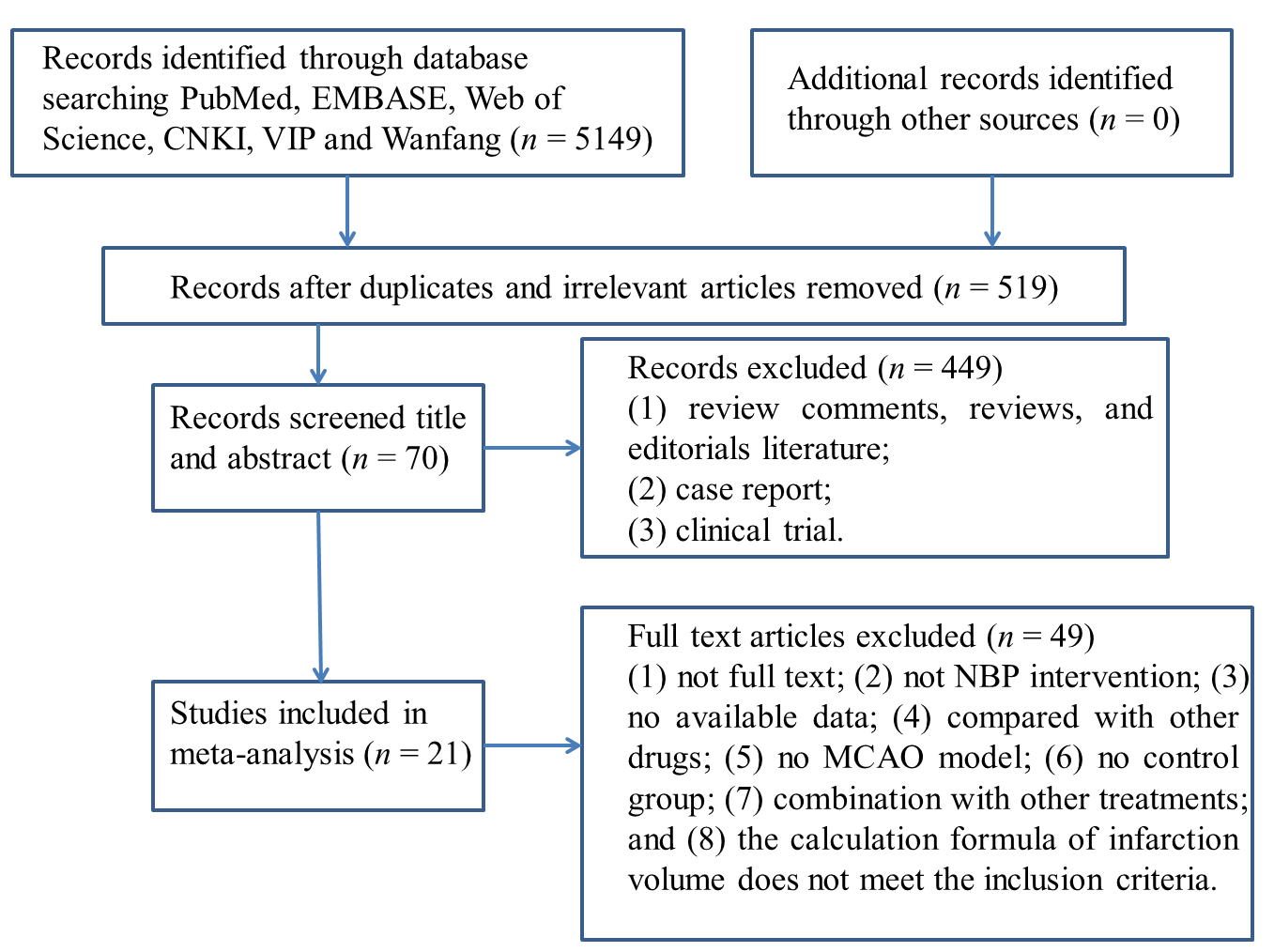
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Grade B (Very good): 0

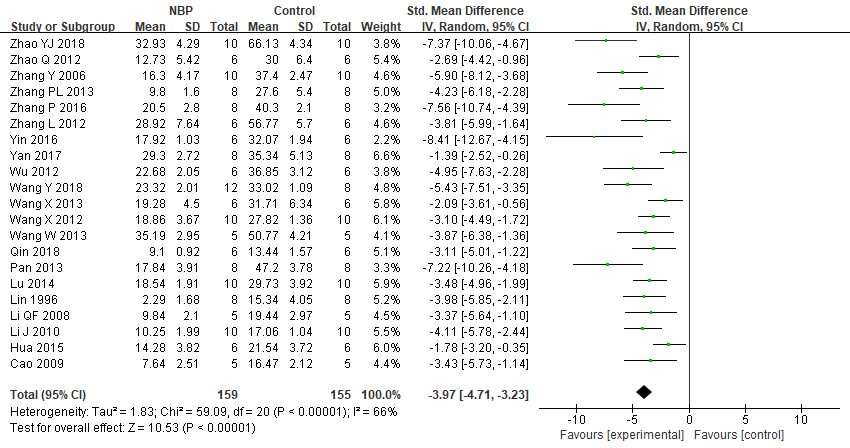
Grade C (Good): C

Grade D (Fair): 0

Grade E (Poor): 0



**Figure 1 Literature inclusion flow chart.** NBP: Dl-3-n-butylphthalide; MCAO: Middle cerebral artery occlusion.



**Figure 2 Forest plot showing effects of dl-3-n-butylphthalide in decreasing cerebral infarction volume compared with the control group.**

E:\我们的征途是星辰大海\butylphthalide\APS投稿用\Fig 3.tif

**Figure 3 Forest plots showing effects of dl-3-n-butylphthalide in decreasing cerebral infarction volume compared with the control group in transient (A) and permanent (B) middle cerebral artery occlusion model, respectively.**

E:\我们的征途是星辰大海\butylphthalide\APS投稿用\Fig 4.tif

**Figure 4 Forest plots showing effects of dl-3-n-butylphthalide in decreasing cerebral infarction volume compared with the control group in pre- (A) and post-administration (B) model, respectively.**

E:\我们的征途是星辰大海\butylphthalide\APS投稿用\Fig 5.tif

**Figure 5 Funnel plot for evaluation of publication bias and underlying mechanism of dl-3-n-butylphthalide in neuroprotection.** A: Funnel plot evaluation of publication bias for the effects of dl-3-n-butylphthalide on infarction volume; B: The underlying mechanism of dl-3-n-butylphthalide in neuroprotection.

**Table 1 Characteristics of the included studies**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Study (yr)** | **Species/age**  **Sex/weight**  **Number (control group/experimental group)** | **Anesthetic** | **Model** | **Intervention dose**  **Administration method**  **Time point/duration** | **Measurement of infarction volume** | **Outcome index** | ***P*-value** |
| Qin *et al*[8], 2018 | SD rats/8 weeks | 5 % chloral hydrate | tMCAO 2 h | 90 mg/kg, daily | Cresyl violet | 1 Infarction volume | <0.05 |
|  | Male/250-300 g | 400 mg/kg | Reperfusion 7 d | Gavage | Image J | 2 Ameliorate body weight loss | <0.05 |
|  | 6/6 | Intraperitoneally |  | Postoperative/7 d |  | 3 Improve neurological behavior scores | <0.05 |
|  |  |  |  |  |  | 4 Reduce brain atrophy volume | <0.01 |
|  |  |  |  |  |  | 5 Upregulate *PTGIS* and *PTGES*; downregulate *TBXAS 1* | <0.05 |
|  |  |  |  |  |  | 6 Inhibit *REN*, *AGT*, *ACE 1*, and *AGTR 1*; upregulate *RoA* | <0.05 |
|  |  |  |  |  |  | 7 Increase the diameter of middle cerebral artery | <0.05 |
| Zhao *et al*[16], 2018 | CD 1 mice/10-12 wk | 10% chloral hydrate | pMCAO 24 h | 120 mg/kg | 2% TTC | 1 Infarction volume | <0.05 |
|  | Male/27-30 g | 35 mg/g |  |  |  | 2 Improve neurological behavior scores | <0.05 |
|  | 10/10 | Intraperitoneally |  |  |  | 3 Decrease the water content of brain | <0.05 |
|  |  |  |  |  |  | 4 Decrease the permeability of blood-brain barrier | <0.05 |
|  |  |  |  |  |  | 5 Decrease pinocytotic vesicles of capillary endothelial cells | |
|  |  |  |  |  |  | 6 Downregulate *MMP 9* | <0.05 |
|  |  |  |  |  |  | 7 Upregulate *Claudin 5*, *VEGF*, *GFAP*, *Nrf 2*, and *HO 1* | <0.05 |
| Wang *et al*[18], 2018 | SD rats/unknown | Unknown | tMCAO 2 h | 1 mg/kg | 2% TTC | 1 Infarction volume | >0.05 |
|  | Male and female/250-280 g |  | Reperfusion 48 h | Intravenously | Image Pro Plus | 2 Improve neurological behavior scores | >0.05 |
|  | 8/12 |  |  | Postoperative/4 h and 24 h |  |  |  |
| Yan *et al*[17], 2017 | SD rats/adult | Unknown | tMCAO 2 h | 75 mg/kg, daily | 2% TTC | 1 Infarction volume | <0.01 |
|  | Male/180-220 g |  | Reperfusion 24 h | Gavage |  | 2 Decrease the water content of brain | <0.05 |
|  | 8/8 |  |  | Preoperative/7 d |  | 3 Decrease the permeability of blood-brain barrier | <0.01 |
|  |  |  |  |  |  | 4 Decrease cell apoptosis |  |
|  |  |  |  |  |  | 5 Decrease ROS, cleaved caspase-3, and p-p38; increase SOD | <0.01 |
|  |  |  |  |  |  | 6 Decrease MDA and p-JNK | <0.05 |
| Zhang *et al*[19], 2016 | SD rats/unknown | Sodium pentobarbital | tMCAO 1 h | 4.5 mg/kg | TTC | 1 Infarction volume | <0.01 |
|  | Male/250-320 g | 50 mg/kg | Reperfusion 24 h | Intraperitoneally |  | 2 Improve neurological behavior scores | <0.001 |
|  | 8/8 |  |  | Postoperative/- |  | 3 Decrease the water content of brain | <0.05 |
|  |  |  |  |  |  | 4 Upregulate *HGF*; downreglate *TLR4* | <0.001 |
| Yin *et al*[20], 2016 | SD rats/unknown | Chloral hydrate | tMCAO 2 h | 80 mg/kg, daily | 2% TTC | 1 Infarction volume | <0.001 |
|  | Male/280-320 g | 300 mg/kg | Reperfusion 24 h | Gavage | Image Pro Plus | 2 Improve neurological behavior scores | <0.05 |
|  | 6/6 | Intraperitoneally |  | Preoperative/7 d |  | 3 Decrease the water content of brain | <0.01 |
|  |  |  |  |  |  | 4 Decrease MDA | <0.01 |
|  |  |  |  |  |  | 5 Increase SOD | <0.05 |
|  |  |  |  |  |  | 6 Increase GSH-Px | <0.001 |
| Hua *et al*[21], 2015 | SD rats/57-61 days | Chloral hydrate | tMCAO 2 h | 60 mg/kg | 2% TTC | 1 Infarction volume | <0.05 |
|  | Male/250-280 g | 300 mg/kg | Reperfusion 24 h | Gavage | Image Pro Plus | 2 Improve neurological behavior scores | >0.05 |
|  | 6/6 | Intraperitoneally |  | Postoperative/- |  | 3 Decrease the water content of brain | <0.01 |
|  |  |  |  |  |  | 4 Decrease MDA; increase GSH, SOD, Nrf 2, Trx, and Bcl-2 | <0.01 |
|  |  |  |  |  |  | 5 Decrease NF-κB p65; increase Txnip | <0.05 |
| Lu *et al*[22],2014 | C57BL6/J/adult | Isoflurane | tMCAO 0.75 h | 10 mg/kg | Cresyl violet | 1 Infarction volume | <0.05 |
|  | Male/unknown | Initiated 3% | Reperfusion 23 h | Intravenous | Image J | 2 Improve neurological behavior scores | <0.05 |
|  | 10/10 | Maintained 1.5% |  | Preoperative/- |  | 3 Decrease MMP 9; increase TIMP 1 | <0.01 |
|  |  |  |  |  |  | 4 Increase SBP and p-ERK | <0.01 |
| Wang *et al*[23], 2013 | SD rats/unknown | Chloral hydrate | tMCAO 2 h | 80 mg/kg, daily | TTC | 1 Infarction volume | <0.05 |
|  | Male/280-320 g | 300 mg/kg | Reperfusion 24 h | Gavage |  | 2 Improve neurological behavior scores | >0.05 |
|  | 6/6 | Intraperitoneally |  | Preoperative/7 d |  | 3 Decrease the water content of brain | >0.05 |
| Wang *et al*[24], 2013 | SD rats/unknown | 10% chloral hydrate | tMCAO 2 h | 80 mg/kg, daily | 2% TTC | 1 Infarction volume | <0.05 |
|  | Male/250 ± 20 g (*n* = 30) | 3 mL/kg | Reperfusion 24 h | Gavage |  | 2 Downregulate *GRP78* and *CHOP* | <0.05 |
|  | 5/5 | Intraperitoneally |  | Preoperative/7 d |  |  |  |
| Pan *et al*[25], 2013 | Wistar rats/unknown | 10% chloral hydrate | pMCAO 24 h | 0.8 g/kg, daily | 2% TTC | 1 Infarction volume | <0.05 |
|  | Male/200-250 g | 3 mL/kg |  | Gavage | Biosens Digitan Image | 2 Decrease the water content of brain | <0.05 |
|  | 8/8 | Intraperitoneally |  | Preoperative/14 d |  | 3 Decrease Smac and S100B | <0.05 |
| Zhang *et al*[26], 2013 | SD rats/unknown | 6 % chloral hydrate | tMCAO 2 h | 200 mg/kg | 2% TTC | 1 Infarction volume | <0.01 |
|  | Male/250 ± 20 g |  | Reperfusion 24 h | Intraperitoneally | Image Pro Plus | 2 Improve neurological behavior scores | <0.01 |
|  | 8/8 | Intraperitoneally |  | Postoperative/- |  | 3 Increase VEGF | <0.01 |
| Wang *et al*[27], 2012 | SD rats/unknown | Chloral hydrate | tMCAO 2 h | 90 mg/kg, daily | 2% TTC | 1 Infarction volume | <0.05 |
|  | Male/250-300 g | 300 mg/kg | Reperfusion 3 d | Gavage |  | 2 Improve neurological behavior scores | <0.05 |
|  | 10/10 | Intraperitoneally |  | Postoperative/3 d |  | 3 Decrease the water content of brain | <0.05 |
|  |  |  |  |  |  | 4 Decrease the ratio of TXB2/6-keto-PGF1α | <0.05 |
| Wu *et al*[28], 2012 | SD rats/unknown | Chloral hydrate | tMCAO 2 h | 80 mg/kg, daily | 2% TTC | 1 Infarction volume | <0.001 |
|  | Male/250-280 g | 350 mg/kg | Reperfusion 24 h | Gavage |  | 2 Improve neurological behavior scores | <0.001 |
|  | 6/6 | Intraperitoneally |  | Preoperative/7 d |  | 3 Decrease the water content of brain | >0.05 |
|  |  |  |  |  |  | 4 Decrease MDA; increase SOD | <0.001 |
| Zhang *et al*[29], 2012 | Wistar Kyoto rats/3 mo | Ketamine, xylazine | pMCAO 7 d | 80 mg/kg, daily | 2% TTC | 1 Infarction volume | <0.01 |
|  | Male/unknown | 75 mg/kg, 10 mg/kg |  | Gavage | Image Pro Plus |  |  |
|  | 6/6 | Intraperitoneally |  | Postoperative/7 d |  |  |  |
| Zhao *et al*[30], 2012 | SD rats/unknown | Chloral hydrate | tMCAO 2 h | 50 mg/kg, daily | 2% TTC | 1 Infarction volume | <0.05 |
|  | Male/250-300 g | 300 mg/kg | Reperfusion 3 d | Gavage |  | 2 Improve neurological behavior scores | <0.05 |
|  | 6/6 | Intraperitoneally |  | Postoperative/3 d |  |  |  |
| Li *et al*[31], 2010 | 129S2/Sv/adult | 4 % chloral hydrate | pMCAO 24 h | 100 mg/kg | 2% TTC | 1 Infarction volume | <0.05 |
|  | Male/20-25 g |  |  | Intraperitoneally |  | 2 Decrease cleaved-caspase 3; caspase 9, p-JNK, and p-p38 | <0.05 |
|  | 10/10 | Intraperitoneally |  | Postoperative 1 h/- |  | 3 Reduce mitochondrial release of cytochrome c and AIF | <0.05 |
| Cao *et al*[32], 2009 | SD rats/3-4 mo | 10% chloral hydrate | tMCAO 2 h | 25 mg/kg, twice a day | TTC | 1 Infarction volume | <0.01 |
|  | Male/280-350 g |  | Reperfusion 3 d | Gavage | Image Pro Plus | 2 Improve neurological behavior scores | <0.05 |
|  | 5/5 | Intraperitoneally |  | Postoperative/3 d |  | 3 Upregulate *VEGF* and *bFGF* | <0.05 |
| Li *et al*[33], 2008 | SD rats/3-4 mo | Unknown | pMCAO 3 d | 25 mg/kg, twice a day | TTC | 1 Infarction volume | <0.05 |
|  | Male/280-350 g |  |  | Gavage | Image Pro Plus | 2 Improve neurological behavior scores | <0.05 |
|  | 5/5 |  |  | Postoperative/3 d |  | 3 Upregulate *VEGF* and *bFGF* | <0.05 |
| Zhang *et al*[34], 2006 | SD rats/unknown | 3% isoflurane | tMCAO 2 h | 10 mg/kg | 4% TTC | 1 Infarction volume | <0.001 |
|  | Male/270-330 g | Endotracheal intubation | Reperfusion 24 h | Intravenously | SPOT Biometrics | 2 Improve neurological behavior scores | <0.01 |
|  | 10/10 |  |  | Intraoperative/- |  |  |  |
| Lin *et al*[35], 1996 | Wistar rats/unknown | Chloral hydrate | pMCAO 24 h | 240 mg/kg | 4% TTC | 1 Infarction volume | <0.001 |
|  | Male/250-350 g |  |  | Gavage |  | 2 Improve neurological behavior scores | <0.001 |
|  | 8/8 |  |  | Postoperative/- |  |  |  |

*PTGIS*: Prostacyclin synthase; *PTGES*: Prostaglandin E synthase; *TBXAS 1*: Thromboxane A2 synthase 1; *REN*: Renin; *AGT*: Angiotensinogen; *ACE 1*: Angiotensin converting enzyme 1; *AGTR 1*: Angiotensin II receptor type 1; tMCAO: Transient middle cerebral artery occlusion; pMCAO: Permanent middle cerebral artery occlusion; TTC: 2,3,5-triphenyltetrazolium chloride; *MMP 9*: Matrix metallopeptidase 9; *VEGF*: Vascular endothelial growth factor; *GFAP*: Glial fibrillary acidic protein; *Nrf 2*: NF-E2-related factor 2; *HO 1*: Heme oxygenase 1; ROS: Reactive oxygen species; SOD: Superoxide dismutase; MDA: Malonaldehyde; *HGF*: Hepatocyte growth factor; *TLR4*: Toll like receptor 4; GSH-Px: Glutathione peroxidase; GSH: Glutathione; Trx: Thioredoxin; Txnip: Thioredoxin-interacting protein; TIMP 1: Tissue inhibitor of metalloproteinase 1; SBP: Spectrin breakdown product; *GRP 78*: Glucose-regulated protein 78; CHOP: C/EBP-homologous protein; Smac: Second mitochondria-derived activator of caspases; S100B: S100 calcium binding protein B; TXB2: Thromboxane B2; 6-keto-PGF1α: 6-keto-prostaglandin F1α; AIF: Apoptosis-inducing factor; *bFGF*: Basic fibroblast growth factor.

**Table 2 Risk of bias of the included studies**

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Study (yr)** | **A** | **B** | **C** | **D** | **E** | **F** | **G** | **H** | **I** | **J** | **Score** |
| Qin *et al*[8], 2018 | √ | √ | √ |  |  |  | √ | √ | √ | √ | 7 |
| Zhao *et al*[16], 2018 | √ | √ | √ |  |  |  |  |  | √ | √ | 5 |
| Wang *et al*[18], 2018 | √ |  | √ | √ |  |  |  | √ | √ |  | 5 |
| Yan *et al*[17], 2017 | √ |  |  | √ |  |  |  |  | √ | √ | 4 |
| Zhang *et al*[19], 2016 | √ |  |  | √ |  |  |  |  | √ | √ | 4 |
| Yin *et al*[20], 2016 | √ | √ | √ |  |  |  |  | √ | √ | √ | 6 |
| Hua *et al*[21], 2015 | √ | √ | √ | √ |  |  |  | √ | √ | √ | 7 |
| Lu *et al*[22], 2014 | √ | √ | √ | √ |  |  |  | √ | √ | √ | 7 |
| Wang *et al*[23], 2013 | √ | √ | √ |  |  |  |  |  | √ | √ | 5 |
| Wang *et al*[24], 2013 | √ | √ | √ |  |  |  |  | √ |  | √ | 5 |
| Pan *et al*[25], 2013 | √ | √ | √ |  |  |  |  | √ |  | √ | 5 |
| Zhang *et al*[26], 2013 | √ | √ | √ |  |  |  |  |  | √ | √ | 5 |
| Wang *et al*[27], 2012 | √ | √ | √ |  |  |  |  |  | √ | √ | 5 |
| Wu *et al*[28], 2012 | √ | √ | √ |  |  |  |  |  | √ | √ | 5 |
| Zhang *et al*[29], 2012 | √ | √ | √ | √ |  |  |  | √ | √ |  | 6 |
| Zhao *et al*[30], 2012 | √ | √ | √ | √ |  |  |  | √ | √ | √ | 7 |
| Li *et al*[31], 2010 | √ |  |  |  |  |  |  |  | √ | √ | 3 |
| Cao *et al*[32], 2009 | √ | √ | √ | √ |  |  | √ | √ |  | √ | 7 |
| Li *et al*[33], 2008 | √ | √ | √ | √ |  |  |  | √ |  | √ | 6 |
| Zhang *et al*[34], 2006 | √ | √ | √ | √ |  |  |  |  | √ | √ | 6 |
| Lin *et al*[35], 1996 | √ |  |  |  |  |  |  |  | √ | √ | 3 |

A: Baseline characteristics; B: Allocation concealment; C: Sequence generation; D: Random housing; E: Blind feeding; F: Random outcome assessment; G: Result evaluator blindness; H: No incomplete outcome data; I: No selective outcome reporting; J: No other sources of bias.