Therapeutic effects of different treatment periods against ischemic stroke and toxicology study of Angong Niuhuang Pill in rats

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Research article

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Abstract

Background: Angong Niuhuang Pill (ANP) is one of the most famous drugs to treat stroke in China, but there is no definite treatment period in drug instruction. In this study, we used middle cerebral artery occlusion (MCAO) model to evaluate its therapeutic effects of different treatment periods and studied its toxic effect in rats.

Methods: Protective effect of ANP was observed in the cerebral ischemia-reperfusion model in rats; ANP (270 mg/kg) three different treatment period included 1 day, 4 days and 7 days. The observation period was 30 days. Therapeutic effect was evaluated by detecting neurological function, cerebral infraction volume, brain histology and cytokines. Three dose including 550, 1640, 4910 mg/kg were studied in toxicology study. The administration period was 30 days. Toxic effect was evaluated by detecting appearance, behavior, excrement character, food-intake, body weight, hematological parameters and biomarkers such as TBA, GST\textsubscript{α}, Cystatin C, clusterin, GSH, S-100B and MBP.

Results: Seven days treatment period of ANP had better effect than 1 day and 4 days treatment periods in rat MCAO model from neurological function scores, the volume of cerebral infarction, brain histology and the serum content of IL-1\textbeta, TNF-\alpha and NO; the brain content of IL-1\textbeta and NO. The results of 30 days multiple dose toxicology study showed no animal death in all groups; in ANP 4910 mg/kg group, the kidney and liver coefficient increased about 10%, the body weight grew more slowly, the TBA increased slightly. There was no abnormal change in histology. These all recovered after drug withdraw for 8 weeks.

Conclusion: Seven days treatment period of ANP had more protective effect than 1 day and 4 days treatment periods in ischemic stroke rat. No observed adverse effect level (NOAEL) of ANP was 1640 mg/kg; the safety margin of ANP was 270-1640 mg/kg. These data provided reference to modify drug instruction.

Background

Angong Niuhuang Pill (ANP) is one of the most famous cerebral ischemia drugs in Chinese traditional medicine. It is treated for stroke. Stroke is a common disease which threatens the health of middle-aged and elderly people, it occurs frequently [1]. Strokes can be classified into two major categories: ischemic and hemorrhagic [2], ischemic stroke is up to 85%, focal cerebral ischemia accounts for 80% of ischemic stroke [3]. Data shows that acute cerebral ischemia is one of the most common cerebrovascular diseases. Currently thrombolysis is the only effective treatment approved for ischemic stroke. However, there are only 3–5% of patients can be selected to do this treatment [4].

It is reported that the main constituent herbs of ANP have many pharmacological effects such as anti-inflammatory, antiplatelet, promotion blood circulation et al. [5] They can improve limb function and accelerate the recovery of hemiplegia after cerebral ischemia.
According to drug instruction, the dosage of ANP is one pill per day, but there is no description about treatment period. Clinical trial reports showed there were four treatment periods of ANP, 1 day, 2 days, 4 days or 7 days. The pharmacological and toxicological study about the rational period of ANP hasn’t been done.

So this paper studied the therapeutic effects of three different treatment periods of ANP and studied toxic effect in order to provide nonclinical reference data for drug instruction modification.

**Methods**

**Reagents**

Angong Niuhuang Pill (ANP) was obtained from Guangzhou Baiyunshan Zhongyi Pharmaceutical Company Limited (Guangzhou, Guangdong, China). Aspirin effervescent tablet was obtained from AstraZeneca Pharmaceutical Co Ltd (Wuxi, Jiangsu, China). Rat IL-1β, TNFα, S100B, MBP, GST-a, clusterin ELISA kits were obtained from Wuhan Elabscience Biotechnology Co., Ltd (Wuhan, Hubei, China). The nitric oxide test kit and GSH assay kit were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). Anti-IL-1β antibody (ab9787) and Anti-TNFα antibody (ab6671) were obtained from Abcam (Cambridge, UK). Blood biochemical reagents (ALT, AST, ALP, TP, ALB, GLB, UREA, Crea, GLU, CHOL, TBil, CK, LDH, P, GGT, TG, Cystatin C, TBA, CA) were obtained from Zhejiang Erkn Biological Technology Co., Ltd (Wenzhou, Zhejiang, China). Blood biochemical reagents (Na+, K+, Cl-) were obtained from MEDICA (USA). PT, APTT, Fbg and TT test kits were obtained from Siemens (Germany). Hematology Reagents (EPK, FFD, FFS, SLS, FBA, RED) were obtained from Sysmex (Japan). Chemical Urinalysis Strips were obtained from URIT (Guilin, Guangxi, China).

**Animals**

Seven-week-old SPF grade male Sprague-Dawley (SD) rats (weighing 240-300 g) were provided from Hunan SJA Animal Company. Rats were housed in SPF animal room of Guangzhou General Pharmaceutical Research Institute Company (GPRI) Center for Drug Non-Clinical Evaluation and Research. The housing environment was maintained at 20-26 °C, humidity 40-70%. Rats were kept in a 12 h light/12 h dark cycle. Rats were given free access to food and water. At the end of study, all animals were euthanized. All animal welfare and experimental procedures were in strict accordance with the Guide for the Care and Use of Laboratory Animals (ethical code number: IA-PD2017005-01, IA-AP2015010-02M; approval date: 04/05/2017, 12/01/2015).

**MCAO model preparation and reperfusion**

Rats were anesthetized by using intraperitoneal (IP) injection of chloral hydrate. Transient focal cerebral ischemia was induced through middle cerebral artery occlusion [6]. A midline neck incision was made, and then the right common carotid artery (CCA) and its branches were isolated. A paraffin-coated nylon monofilament was inserted from CCA to internal carotid artery (ICA) and sent toward the origin of middle
cerebral artery (MCA) to produce focal cerebral ischemia. The middle cerebral artery was blocked for 2 hours and reperfusion was conducted for 3 hours.

**Neurological function evaluation**

Neurological deficit scores (NDS) were assessed according to the ZeaLonga method [7] at 3 h, D7, D14, D21, D28 after ischemia/reperfusion. Using a 5-point scale as follows, score 0, no neurological deficit; score 1, mild focal neurological deficit (with contralateral forelimb flexion); score 2, moderate focal neurological deficit (circling to the contralateral side); score 3, severe focal neurological deficit (falling to the contralateral side); score 4, no spontaneous activity with a depressed level of consciousness or death. Only the rats with score of 1-3 at 3 h were considered successful models.

**Group and dose**

The model rats were randomly divided into 6 groups as followed: Sham group, model group, positive drug aspirin 25 mg/kg (7 days) group, ANP 270 mg/kg (1 day, 4 days and 7 days) groups. And animals were fed for 30 days.

**Cerebral infraction volume analysis**

Rats were anesthetized with 20% urethane. Six rats in each group were randomly selected to collect brain to do 2,3,5-triphenyltetrazolium chloride (TCC) staining. Brain was frozen at -20 °C for 30 minutes and cut into 2 mm thick slices using metal brain matrix. And then TCC staining was done to analyze cerebral infraction area. Slides were incubated in 1% TTC in PBS for 10 minutes at 37 °C in dark. The infarct region appeared in white color while the normal brain appeared in red color. Infarct size (mm$^2$) was determined by digital planimetry of the slices using Image-Pro software (Image-Pro Plus 6.0) (Media Cybernetics Inc.). Infarct volume (mm$^3$) was corrected by section interval thickness. Percentage of cerebral infarction volume = infarct volume / total brain volume × 100%.

**Brain histology analysis**

Brains were immersed in 4 % paraformaldehyde and stained with hematoxylin-eosin (HE). The tissue was embedded in paraffin after dehydration and coronal slices were sectioned. Tissue structure was observed under common microscope and scored according to the degree of injury. “-” there was no edema in the cerebral cortex, and the nerve cells were normal; “±” there was edema in the cerebral cortical nerve cells and small fraction of neuronal necrosis, and the infarction area was not more than 1/6 of the left cortex area; “+” there was edema in the cerebral cortical nerve cells and small fraction of neuronal necrosis, and the infarction area was not more than 1/3 of the left cortex area; “++” there was edema in the cerebral cortical nerve cells and large fraction of neuronal necrosis, and the infarction area was not more than 1/3–2/3 of the left cortex area; “+++” there was edema in the cerebral cortical nerve cells and large fraction of neuronal necrosis, and the infarction area was more than 2/3 of the left cortex area.

**Cytokine detection**
Serum cytokine detection: The whole blood was incubated for 2 hours at room temperature and then centrifuged for 15 minutes (4 °C, 3000 rpm). The serum content of IL-1β, TNFα and NO were detected using the ELISA kits or biochemical kits. Detection was done in accordance to the kit manufacturer recommendations. The absorbance was measured at 450 nm (IL-1β, TNFα) or 550 nm (NO) wavelength using an ELx800 type microplate reader (BioTek, America).

Brain cytokine detection: brain slices were done with immunohistochemistry assays. Slides were deparaffinized, rehydrated, and incubated with 3% H$_2$O$_2$ for 10 minutes to inactivate endogenous peroxidases. Slides were drop normal goat serum sealing uid for 30 minutes. Then 1:50 dilution of rabbit anti-IL-1β and 1:100 dilutions of rabbit anti-TNFα polyclonal antibodies (abcam) were added at 4°C overnight. After washing, the slices were immersed in poly-peroxidase-anti-mouse/rabbit IgG (Elabsicence) for 30 minutes at 37°C. Finally, slices were incubated with DAB solution (Elabsicence) at 20-37°C for 10–15 minutes. Images were obtained using a Leica DM 4000B photo microscope. The IL-1β and TNFα staining intensity were scored as 0 (negative), 1+ (weak positive), 2+ (mild positive), 3+ (medium positive) and 4+ (strong positive).

**Toxicology study**

ANP used in pharmacology study was up to 7 days; therefore, 30 days repeated dose toxicity study was designed to assess the drug safety. For detail, refer to ICH S4 “duration of chronic toxicity testing in animals”.

Rats were randomly divided into 4 groups according body weight, control group, ANP (550 mg/kg, 1640 mg/kg and 4910 mg/kg) groups. 4 weeks and 6 weeks for recovery were followed. Detection indicators were as follows: appearance, behavior, excrement character were observed every day. Body weight was measured weekly. Food intake was measured weekly. Hematology, blood biochemistry, urine, bone marrow, and histopathology were detected. Biomarkers such as TBA, GSTα, Cystatin C, clusterin, GSH, S-100B and MBP were also detected.

**Statistical analysis**

Statistical analysis was performed using SPSS software (18.0 versions, SPSS, Chicago, IL, USA), all the data are presented as mean ± SD or mean ± SEM. Statistical significance was calculated using a Dunnett’s test. One-way ANOVA was used to compare multiple sets of data. Ranked ordinal data used Ridit test and u-test. The significance level was set at $P < 0.05$.

**Results**

**ANP improved rat’s neurological function after MCAO**

Compared with model group, the neurological function scores of ANP 1 day, 4 days and 7 days groups were decreased significantly ($P < 0.01$ or $P < 0.05$). The neurological function score of aspirin group was
decreased ($P < 0.01$). The result was shown in Fig.1.

**ANP reduced the volume of cerebral infarction after MCAO**

Compared with model group, the percentage of cerebral infarct volume of ANP 1 day and 7 days groups were decreased ($P < 0.05$). The percentage of cerebral infarct volume of aspirin group was decreased ($P < 0.01$). The result was shown in Fig.2.

**Protective effect of ANP on brain histopathology after MACO**

The pathological changes of the cortex in brain tissue of focal cerebral ischemia reperfusion rat models were observed and scored according to the degree of injury. Compared with model group after *Ridit test*, the R values of ANP 1 day, 4 days and 7 days groups were decreased ($P < 0.05$ or $P < 0.01$) significantly. The R value of aspirin group was decreased ($P < 0.01$). The result was shown in Fig.3 and Tab.1.

**ANP decreased cytokines in serum and brain tissues after MACO**

Serum cytokine: Compared with model group, the IL-1$\beta$ and NO content of ANP 1 day, 4 days and 7 days groups were decreased ($P < 0.05$ or $P < 0.01$). The IL-1$\beta$, TNF$\alpha$ and NO of aspirin group were decreased ($P < 0.01$). The result was shown in Fig.4.

Brain cytokine: Compared with model group, the IL-1$\beta$ and TNF$\alpha$ content of ANP 1 day and 7 days group were decreased ($P < 0.05$ of or $P < 0.01$). The TNF$\alpha$ and NO of aspirin group was decreased ($P < 0.01$ or $P < 0.05$). The results were shown in Fig.5, Fig.6, Fig.7, Tab.2 and Tab.3.

**Results of toxicological study**

**General observation**

There was no abnormal behavior of all animals after dose.

**Body weight**

Compared with normal group, body weight of male rats of ANP 4910 mg/kg group was decreased ($P < 0.05$) after dose, it can recover after drug withdrawal for 4 weeks. The result was shown in Fig.8.

**Food intake**

There was no significance difference between ANP groups and normal group ($P > 0.05$) after dose.

**Hematological examination**

There was no significance difference between ANP groups and normal group ($P > 0.05$) after dose.

**Blood biochemical examination**
There was no significance difference between ANP groups and normal group ($P > 0.05$) after dose.

**Ophthalmological examination**

There was no significance difference between ANP groups and normal group ($P > 0.05$) after dose.

**Urinary examination**

There was no significance difference between ANP groups and normal group ($P > 0.05$) after dose.

**Organs coefficient examination**

Compared with normal group, liver organ coefficient of female rats of ANP 4910 mg/kg group was increased ($P < 0.05$) after dose; it can recover after drug withdrawal for 4 weeks. Compared with normal group, kidney organ coefficient of male rats of ANP 4910 mg/kg groups were increased ($P < 0.05$) after dose; it can recover after drug withdrawal for 6 weeks. The results were shown in Tab.4.

**Biomarkers detection**

Compared with normal group, the content of TBA of male rats of ANP 4910 mg/kg group was increased ($P < 0.05$) after dose; it can recover after drug withdrawal for 4 weeks. The result was shown in Tab.5.

**Histology examination**

There was no significant change related to the subjects after dose and drug withdrawal.

**Discussion**

ANP is a treasure of Chinese traditional medicine with a long history, it has remarkable curative effect. It is widely used in clinical treatment of central nervous system diseases in China, typically like stroke. However, the course of treatment is not specified in drug instruction, so it is controversial about its treatment period. In this context, this paper was done to compare the effect of three different courses on MACO rats, which were 1 day, 4 days and 7 days.

The results showed that the effect of 7 days treatment period was better than 1 day or 4 days treatment period. After 7 days of treatment, comparing with model group, the neurological deficit scores of ANP group was reduced; the cerebral infarction volume was reduced; the degree of degeneration and necrosis was lower in nerve tissue.

In this paper, we also explored the effect mechanism of ANP. The mechanism of cerebral ischemia is complex, involving multiple aspects of pathology and physiology, such as excitatory amino acid toxicity, apoptosis, oxidative stress, intracellular calcium overload etc. [8] Among them, inflammatory reaction is the most important damage mechanism [9]. TNFα and IL1β play important roles in this process. TNFα is one of the nuclear factor signal transduction targets, and has a variety of immunomodulatory effects,
which can mediate the release of various inflammatory factors such as IL1 and IL6, and participate in various pathophysiological processes such as infection and tissue repair. Over expression of TNFα during cerebral ischemia can lead to a variety of pathological changes [10]. IL1β is an inflammatory cytokine that stimulates monocyte cellular activation and infiltration, glial activation, neuronal and myelin loss, blood-brain barrier perturbation, and further persistent inflammation [11-13]. Cerebral ischemia will cause the activation of microglia and astrocytes [14]. Proliferating microglia and astrocytes release a large number of pro-inflammatory mediators and neurotoxic molecules, such as IL1β, which involved in the inflammatory response induced by cerebral ischemia [15]. Our results indicate that ANP could affect the most important factors involved in pro-inflammatory responses in ischemia tissue thereby ameliorating the ischemia-reperfusion mediated brain dysfunction.

Nitric oxide (NO) is an important second messenger with multiple functions involved in the control of vasomotor tone, vascular homeostasis, neuronal, and immunological functions [16]. Low concentration of NO considered as protective to brain vasculature by inducing vasodilatation and improving blood flow to penumbra [17, 18]. However, during the ischemia-reperfusion stage, the activation of iNOS leads to the excessive release of NO, which causes the reaction of NO to superoxide (O₂⁻) generating peroxynitrite radicals (ONOO⁻) and exacerbation of brain ischemia and reperfusion injury damage [17, 19-21]. Our results indicate that ANP could down regulate the NO level of brain tissue and serum, could inhibit NO generation, and decrease the negative effect of NO to nerve system.

According to ICH S4 “duration of chronic toxicity testing in animals”, 30 days multiple dose toxicological study was done to support clinical 7 days use. The results showed that the no observed adverse effect level (NOAEL) of ANP was 1640 mg/kg, which was 6 times of the pharmacological dose. The dose of 4910 mg/kg of ANP could cause slow weight growth and TBA increasing, rose liver and kidney organ coefficients about 10%. These could recover after 6 weeks of drug withdrawal. The potential toxic target organ was liver and kidney.

**Conclusion**

Seven days treatment period of ANP had more protective effect than 1 day and 4 days treatment periods in ischemic stroke rat. No observed adverse effect level (NOAEL) of ANP was 1640 mg/kg; the safety margin of ANP was 270-1640 mg/kg. These data provided reference to modify the drug instruction.

**Abbreviations**

ALB: Albumin; ALP: Alkaline phosphatase; ALT: Alanine transaminase; ANP: Angong Niuhuang Pill; AST: Aspartate aminotransferase; CA: Calcium; CCA: Common carotid artery; CHOL: Total cholesterol; CK: Creatine kinase; Crea: Creatinine; GGT: Gamma-glutamyl transpeptidase; GLB: Globulin; GLU: Glucose; GSH: Glutathione; GSTα: Glutathione S-transferase; ICA: Internal carotid artery; iNOS: Inducible nitric oxide synthase; LDH: Lactate dehydrogenase; MBP: Myelin basic protein; MCA: Middle cerebral artery; MCAO: Middle cerebral artery occlusion; NDS: Neurological deficit scores; NO: Nitric oxide; P: Phosphorus; S-
100B: S100 calcium binding protein B; TBA: Total bile acid; Tbil: Total bilirubin; TCC: 2,3,5-triphenyltetrazolium chloride; TG: Triglycerides; TP: Total Protein.

**Declarations**

**Ethics approval and consent to participate**

All animal welfare and experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Center. The ethical code numbers were IA-PD2017005-01 and IA-AP2015010-02M.

**Consent for publication**

Not applicable.

**Availability of data and materials**

The data used and/or analyzed during the current study available from the corresponding author on reasonable request.

**Competing interests**

The authors declare that they have no any competing interests.

**Funding**

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**Authors’ contributions**

JZC and YSC contributed equally to this work, participated in the study design, analyzed the data and wrote the manuscript. QPG designed the experiment and wrote the manuscript. YFH, JSH, TW, YL, ZLP, WJL and NN carried out the experiments, and made suggestions on the manuscript. All authors read and approved the final manuscript.

**Acknowledgements**

Not applicable.

**References**


### Tables

**Tab.1** Effect on the pathological changes of the cortex in brain tissue of focal cerebral ischemia reperfusion rat models. *#P* < 0.05, **##P* < 0.01 relative to the sham group.*P* < 0.05, **##P* < 0.01 relative to the model group.

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**Tab.2** Effect on the IL1β changes of the cortex in brain tissue of focal cerebral ischemia reperfusion rat models. *#P* < 0.05, **##P* < 0.01 relative to the sham group.*P* < 0.05 relative to the model group.
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**Tab.3** Effect on the TNFα changes of the cortex in brain tissue of focal cerebral ischemia reperfusion rat models. ###<i>P</i> < 0.01 relative to the sham group. *<i>P</i> < 0.05, **<i>P</i> < 0.01 relative to the model group.

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**Tab.4** Comparison of liver and kidney organ coefficient parameters among ANP treated groups at doses of 550, 1640 and 4910 mg/kg body weight/day, and the control group rats, during 4 weeks dosing and another 6 weeks for recovery. *<i>P</i> < 0.05, **<i>P</i> < 0.01 relative to the control group.
Tab.5 Comparison of TBA parameters among ANP treated groups at doses of 550, 1640 and 4910 mg/kg body weight/day, and the control group male rats, during 4 weeks dosing and another 6 weeks for recovery. *P < 0.05 relative to the control group.

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Figures
Figure 1

Effect of ANP on neurological function following MCAO using neurological deficit scores (NDS). The diagram shows the behavioral score of each group (n = 12). Values are expressed as the mean ± SEM. *P < 0.05, **P < 0.01 relative to model group.
Figure 2

Infarct volume of rat brains from each treatment group. Representative samples of TTC staining of brain slices in the sham group, model group, aspirin group and ANP 270 mg/kg (1 day, 4 days and 7 days) groups (A). The quantitative analysis of the percentage of infarct volume versus whole area (B). Values are expressed as the mean ± SEM (n = 6). *P < 0.05, **P < 0.01 relative to the model group.
Figure 3
Pathological changes of the cortex in brain tissue of focal cerebral ischemia reperfusion rat models (HE). HE staining assay in sham group (A), model group (B), aspirin group (C), ANP 270 mg/kg (1 day, 4 days, 7 days) groups (D-F).

Figure 4
Effect on the serum IL-1β (A), TNFα (B) and NO (C) levels of focal cerebral ischemia reperfusion rat models (mean ± SD, n = 8). ##P < 0.01 relative to the sham group. *P < 0.05, **P < 0.01 relative to the model group.
Figure 5

Effects of ANP on the expression of IL-1β after MCAO. Immunohistochemistry results in sham group (A), model group (B), aspirin group (C), ANP 270 mg/kg (1 day, 4 days, 7 days) groups (D-F).
Figure 6

Effects of ANP on the expression of TNFα after MCAO. Immunohistochemistry results in sham group (A), model group (B), aspirin group (C), ANP 270 mg/kg (1 day, 4 days, 7 days) groups (D-F).
Figure 7

Effects of ANP on the expression of NO in brain tissue after MCAO (mean ± SD, n = 6). ##P < 0.01 relative to the sham group. *P < 0.05 relative to the model group.
Figure 8

Comparison of body weight change among ANP treated groups, at doses of 550, 1640 and 4910 mg/kg body weight and control group in female (A) and male (B) rats during 4 weeks dosing and another 6 weeks for recovery. Values are expressed as the mean ± SD (D0~W3, n = 25; W4, n = 20; W5~W8, n = 10; W9~W10, n = 5). *P < 0.05, **P < 0.01 relative to the control group.

Supplementary Files

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- NC3RsARRIVEGuidelinesChecklist.pdf