Discussion on rationality of administration of Angong Niuhuang Pills from pharmacological and toxicological perspectives

Jianzhao Chen
Guangzhou General Pharmaceutical Research Institute
Yushuang Chai
Guangzhou Baiyunshan Zhongyi Pharmaceutical Company Ltd.
Yuanfeng He
Guangzhou Baiyunshan Zhongyi Pharmaceutical Company Co Ltd
Jisheng Huang
Guangzhou General Pharmaceutical Research Institute
Ting Wan
Guangzhou Baiyunshan Zhongyi Pharmaceutical Company Ltd.
Yuan Lu
Guangzhou General Pharmaceutical Research Institute
Zilun Peng
Guangzhou General Pharmaceutical Research Institute
Wenjuan Li
Guangzhou General Pharmaceutical Research Institute
Na Ning
Guangzhou Baiyunshan Zhongyi Pharmaceutical Company Ltd.
Qiuping Guo (vipvivenguoguo@126.com)
Guangzhou General Pharmaceutical Research Institute

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Abstract

Background: Investigate the different treatment course of ANP from pharmacology and toxicology to provide scientific basis for clinic use.

Method: In pharmacology study, cerebral ischemia-reperfusion model was made; rats were divided into six groups, Sham, model, aspirin 25 mg/kg, ANP 270 mg/kg (1 day, 4 days and 7 days) groups. Rats were fed for 30 days. Neurological function, cerebral infraction volume, brain histopathology, cytokines were detected; in toxicology study, rats were divided into four groups, normal control, ANP (550, 1640, 4910 mg/kg) group. ANP was daily administered by gavage for 30 days. Detection indicators included appearance, behavior, excrement character, food-intake, body weight, hematological parameters, etc. In addition, biomarkers such as TBA, GSTα, Cystatin C, clusterin, GSH, S-100B and MBP were also detected.

Result: In pharmacology study, compared with model group, the neurological function scores of ANP 270mg/kg (1 day, 4 days and 7 days) were decreased ($P<0.11$ or $P<0.05$); the volume of ANP 270mg/kg (1 day and 7 days) were decreased ($P<0.05$); the R value of ANP 270mg/kg (1 day, 4 days and 7 days) groups were decreased ($P<0.11$ or $P<0.05$); the serum content of IL-1β, TNFα and NO of ANP 270 mg/kg(1 day, 4 days and 7 days) groups were decreased ($P< 0.05$); the brain content of IL-1β and NO of ANP 270 mg/kg(1 day, 4 days and 7 days) groups were decreased ($P<0.05$). In toxicology study, no mortality, ophthalmic abnormalities were identified. Compared with normal control group, body weights were significantly lower in ANP 4910 mg/kg group; TBA was significantly increased in ANP 4910mg/kg group; liver organ coefficient of female rats of ANP 4910 mg/kg group was increased ($P < 0.05$); kidney organ coefficient of male rats of ANP 1640mg/kg, 4910 mg/kg groups were increased ($P < 0.05$), these all recovered after drug withdraw for 8 weeks.

Conclusion: The effect of ANP 270 mg/kg (7 days) was much better than ANP (1 day and 4 days). ANP 550mg/kg is non toxicity dose. So, ANP is taken one pill peer day for 7 days is safety and effective, it can be used as the scientific basis for clinic use.

Background

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].

Most patients who do not have a timely treatment are left with serious sequelae. Angong Niuhuang Pill (ANP) is one of the most famous cerebral ischemia drugs in Chinese traditional medicine. The first description of ANP is in Treatise on Differentiation and Treatment of Epidemic Febrile Diseases, written by Wu Jutong in Qing Dynasty. 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] Its ingredients can improve limb function and accelerate the recovery of hemiplegia after cerebral ischemia. Thus, ANP is mainly used to treat brain diseases, such as cerebral infarction, cerebral hemorrhage, cerebral trauma, viral encephalitis and so on.

The usage and dosage of ANP is one pill per day in accordance with drug instruction, but there is no detail description about the course of treatment. Clinical trial reports show that there are several course of ANP to treat cerebral infarction or cerebral hemorrhage, such as one pill per day for 1 day, 2 days, 4 days or 7 days. No systematic study about the course rationality of ANP has yet been done.

So this paper investigated three different usage of ANP from pharmacology and toxicology in order to provide scientific basis for clinical rational use.

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 obtain from Sysmex (Japan). Chemical Urinalysis Strips were obtained from URIT (Guilin, Guangxi, China).

Animals

Seven-week-old SPF grade male Sprague-Daweley (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 ℃, 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 by our veterinary staff in our laboratory under urethane anesthesia using intravenous KCl after blood collection. All animal welfare and experimental procedures were in strict accordance with the Guide for the Care and Use of Laboratory Animals and related ethical regulations of Center.

Pharmacologic study: Middle cerebral artery occlusion (MCAO) model preparation and reperfusion

Rats were anesthetized by using intraperitoneal (IP) injection of chloral hydrate (300 mg/kg). 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. The sham operation was performed in the same manner except that the nylon thread was not inserted. During surgery, the rats' temperature was kept with an electric heating lamp and intraperitoneal injection of normal saline was taken to prevent dehydration.

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. The score is higher means neurological deficits are more serious. 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 histopathological analysis

Six rats in each group were randomly selected to collect brain to do histopathological analysis and cytokine detection. 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 ºC, 3000 rpm). The serum content of IL-1β, TNFα and NO were detected using the ELISA kits or biochemical kits. Detection was followed 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_2O_2 for 10 minutes to inactivate endogenous peroxidases. Slides were drop normal goat serum sealing fluid 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, multiple dose toxicity study was designed 30 days to assess drug safety7 day’s clinical use. 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

Experiment 1: Protective effects of different usage and dosage of ANP on MACO

Effect of ANP on neurological function evaluation

The neurological function score of sham group was 0, model group were 1.92–2.33. Compared with model group, the scores of ANP 270 mg/kg (1 day, 4 days and 7 days) group were decreased significantly ($P < 0.01$ or $P < 0.05$). The score of aspirin 25 mg/kg (7 days) group was decreased ($P < 0.05$). The result was shown in Fig. 1.

Effect of ANP on cerebral infraction volume

The percentage of infarct volume of sham group was 0, model group was 16.39%. Compared with model group, the infarct volume of ANP 270 mg/kg (1 day and 7 days) group were decreased ($P < 0.05$). The volume of aspirin 25 mg/kg (7 days) group was decreased ($P < 0.05$). The result was shown in Fig. 2.

Effect of ANP on brain Histopathology

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. After Ridit test, the R value of sham group was 0.05, model group was 0.75. Compared with model group, the R value of ANP270 mg/kg (1 day, 4 days and 7 days) group was decreased ($P < 0.05$ or $P < 0.01$) significantly. The R value of aspirin 25 mg/kg (7 days) group was decreased. The result was shown in Fig. 3 and Table 1.

Table 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.

Group	-	±	+	++	+++	$\overline{R}$ Value
Sham	6/6	0/6	0/6	0/6	0/6	0.0500
Model	0/6	0/6	2/6	4/6	0/6	0.7500 ##
Aspirin (25 mg/kg, 7 days)	0/6	2/6	4/6	0/6	0/6	0.3528 ##**
ANP (270 mg/kg, 1 day)	0/6	3/6	3/6	0/6	0/6	0.2958 #**
ANP (270 mg/kg, 4 days)	0/6	0/6	5/6	1/6	0/6	0.5375 ##*
ANP (270 mg/kg, 7 days)	0/6	3/6	2/6	1/6	0/6	0.3667 ##**

Effect of ANP on cytokines in the serum and brain tissues

Serum cytokine: compared with sham group, the content of TNFα, IL-1β and NO were increased greatly (P < 0.05) in model group. Compared with model group, the IL-1β and NO content of ANP 270 mg/kg (1 day, 4 days and 7 days) groups were decreased (P < 0.05). The IL-1β, TNFα and NO of aspirin 25 mg/kg (7 days) group was decreased (P < 0.05). The result was shown in Fig. 4.

Brain cytokine: compared with sham group, the content of IL-1β and NO were increased greatly (P < 0.05) in model group. Compared with model group, the IL-1β content of ANP 270 mg/kg (1 day and 7 days) group were decreased (P < 0.05). The IL-1β and NO of aspirin 25 mg/kg (7 days) group was decreased (P < 0.05). The results were shown in Fig. 5, Fig. 6, Fig. 7, Table 2 and Table 3.

Table 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.

Group	0	1+	2+	3+	4+	$\overline{R}$ Value
Sham	3/6	3/6	0/6	0/6	0/6	0.1148
Model	0/6	1/6	2/6	1/6	2/6	0.6667 ##
Aspirin (25 mg/kg, 7 days)	0/6	3/6	2/6	1/6	0/6	0.4126 #
ANP (270 mg/kg, 1 day)	0/6	3/6	3/6	0/6	0/6	0.3648 *
ANP (270 mg/kg, 4 days)	0/6	2/6	0/6	3/6	1/6	0.6352 ##
ANP (270 mg/kg, 7 days)	0/6	3/6	3/6	0/6	0/6	0.3648 *

Table 3

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

Group	0	1+	2+	3+	4+	$\overline{R}$ Value
Sham	3/6	3/6	0/6	0/6	0/6	0.1000
Model	0/6	0/6	0/6	5/6	1/6	0.8444 ##
Aspirin (25 mg/kg, 7 days)	0/6	3/6	3/6	0/6	0/6	0.3167 **
ANP (270 mg/kg, 1 day)	0/6	0/6	5/6	1/6	0/6	0.5250 ##*
ANP (270 mg/kg, 4 days)	0/6	2/6	1/6	2/6	1/6	0.5694 ##*
ANP (270 mg/kg, 7 days)	1/6	0/6	4/6	1/6	0/6	0.4528 ##**

Experiment 2: Toxicological study of ANP

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

About 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 1640 mg/kg, 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 Table 4.

Table 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.. *P < 0.05, **P < 0.01 relative to the control group.

Group	Liver coefficient (female rats)			Kidney coefficient (male rats)		
	W4	W8	W10	W4	W8	W10
Control	2.636 ± 0.240	2.504 ± 0.188	2.391 ± 0.099	0.655 ± 0.057	0.583 ± 0.025	0.953 ± 0.803
ANP (550 mg/kg)	2.554 ± 0.120	2.666 ± 0.208	2.419 ± 0.051	0.650 ± 0.063	0.600 ± 0.054	0.634 ± 0.066
ANP (1640 mg/kg)	2.763 ± 0.149	2.551 ± 0.205	2.415 ± 0.094	0.718 ± 0.056*	0.589 ± 0.031	0.583 ± 0.074
ANP (4910 mg/kg)	2.918 ± 0.120**	2.581 ± 0.209	2.395 ± 0.246	0.764 ± 0.103*	0.671 ± 0.022**	0.606 ± 0.053

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 withdrawal for 4 weeks. The result was shown in Table 5.

Table 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.

Group	TBA (μmol/L)		
	W4	W8	W10
Control	23.5 ± 11.2	23.4 ± 7.5	21.5 ± 12.5
ANP (550 mg/kg)	30.1 ± 11.9	19.0 ± 4.4	19.3 ± 5.6
ANP (1640 mg/kg)	32.1 ± 15.0	14.8 ± 4.7	17.8 ± 9.6
ANP (4910 mg/kg)	97.4 ± 108.7*	16.0 ± 7.8	22.8 ± 8.3

Histopathological 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 and remarkable curative effect which widely used in clinical treatment of central nervous system diseases in China. It is typically used for treating some diseases like stroke, encephalitis and meningitis. However, the course of treatment is not specified in drug instruction, so the clinical usage of ANP is controversial at present. In this context, our study was done to compare the effect of three different course of ANP, which were 1 day, 4 days and 7 days, and we attempted to identify the safety of these treatment via a 30 days' multiple dose toxicology study so as to provide reference for clinical use.

Pharmacology study results showed that in the acute phase of stroke, continuous use of ANP for 7 days, the efficacy is better than using 1 day or 4 days. ANP showed an obvious therapeutic effect on MCAO rats. It could reduce the neurological deficit scores and cerebral infarction volume; lower the degree of degeneration and necrosis of nerve tissue. The anti-cerebral ischemia effect of ANP appeared to work by reduce serum NO, IL-1β and TNFα.

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] In the midst of them, inflammatory reaction is the most important damage mechanism [9]. TNFα and IL-1β play important roles in this process as two important inflammatory factors. 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 IL-1 and IL-6, 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]. IL-1β 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 IL-1β, 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 (O_2^-) generating peroxynitrite radicals ($ONOO^-$) and exacerbation of brain ischemia-and reperfusion-injury damage [17, 19–21]. ANP

down-regulated the level of brain tissue and serum, suggest that ANP could also inhibit NO generation, decrease the negative effect of NO to nerve system.

The toxicological results showed that the safe dose of ANP for 30 days was 550 mg/kg, which was about 12 times of the clinical dosage and 2 times of the equivalent dose. A dose of 4910 mg/kg of ANP can cause slow weight growth, increased early toxicity marker TBA in liver, and increased liver and kidney organ coefficients by about 10%. This may be an adaptive response to the drug, which could be recovered after 8 weeks of drug withdrawal. The potential toxic target organ was liver and kidney.

Conclusion

After comparison, in the acute phase of stroke, continuous use of ANP for 7 days had significant efficacy; 30 days' multiple dose toxicity test showed that continuous use of 30 days, the safety dose was 550 mg/kg, it means that ANP is taken one pill peer day for 7 days is safety and effective, it can be used as the scientific basis for clinic use.

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 in strict accordance with the Guide for the Care and Use of Laboratory Animals and related ethical regulations of Center. The ethical code number was IA-AP2015010-02M and IA-PD2017005-01.

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.

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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. The other authors carried out the experiments, and made suggestions on the manuscript. All authors read and approved the final manuscript.

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References

1. Collaborators GBDN: **Global, regional, and national burden of neurological disorders, 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016**. *Lancet Neurol* 2019, **18**(5):459-480.
2. Al-Qazzaz NK, Ali SH, Ahmad SA, Islam S, Mohamad K: **Cognitive impairment and memory dysfunction after a stroke diagnosis: a post-stroke memory assessment**. *Neuropsychiatric disease and treatment* 2014, **10**:1677-1691.
3. Altintas O, Altintas MO, Tasal A, Kucukdagli OT, Asil T: **The relationship of platelet-to-lymphocyte ratio with clinical outcome and final infarct core in acute ischemic stroke patients who have undergone endovascular therapy**. *Neurological research* 2016, **38**(9):759-765.
4. Wang N, Zhang Y, Wu L, Wang Y, Cao Y, He L, Li X, Zhao J: **Puerarin protected the brain from cerebral ischemia injury via astrocyte apoptosis inhibition**. *Neuropharmacology* 2014, **79**:282-289.
5. Guo Y, Yan S, Xu L, Zhu G, Yu X, Tong X: **Use of angong niuhuang in treating central nervous system diseases and related research**. *Evidence-based complementary and alternative medicine : eCAM* 2014, **2014**:346918.
6. Vakili A, Kataoka H, Plesnila N: **Role of arginine vasopressin V1 and V2 receptors for brain damage after transient focal cerebral ischemia**. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism* 2005, **25**(8):1012-1019.
7. Longa EZ, Weinstein PR, Carlson S, Cummins R: **Reversible middle cerebral artery occlusion without craniectomy in rats**. *Stroke* 1989, **20**(1):84-91.

8. Guo Y, Xu X, Li Q, Li Z, Du F: **Anti-inflammation effects of picroside 2 in cerebral ischemic injury rats**. *Behavioral and brain functions : BBF* 2010, **6**:43.
9. Pei J, You X, Fu Q: **Inflammation in the pathogenesis of ischemic stroke**. *Front Biosci (Landmark Ed)* 2015, **20**:772-783.
10. Kim AS, Easton JD, Johnston SC: **Risk of Stroke after Transient Ischemic Attack or Minor Stroke**. *The New England journal of medicine* 2016, **375**(4):386-387.
11. Perez-Polo JR, Rea HC, Johnson KM, Parsley MA, Unabia GC, Xu GY, Prough D, DeWitt DS, Paulucci-Holthauzen AA, Werrbach-Perez K *et al*: **Inflammatory cytokine receptor blockade in a rodent model of mild traumatic brain injury**. *Journal of neuroscience research* 2016, **94**(1):27-38.
12. Zhu Y, Liu C, Sun Z: **Early Combined Therapy with Pharmacologically Induced Hypothermia and Edaravone Exerts Neuroprotective Effects in a Rat Model of Intracerebral Hemorrhage**. *Cell Biochem Biophys* 2015, **73**(2):581-587.
13. Mao LL, Hao DL, Mao XW, Xu YF, Huang TT, Wu BN, Wang LH: **Neuroprotective effects of bisperoxovanadium on cerebral ischemia by inflammation inhibition**. *Neuroscience letters* 2015, **602**:120-125.
14. Li Y, Xu XL, Zhao D, Pan LN, Huang CW, Guo LJ, Lu Q, Wang J: **TLR3 ligand Poly IC Attenuates Reactive Astrogliosis and Improves Recovery of Rats after Focal Cerebral Ischemia**. *CNS neuroscience & therapeutics* 2015, **21**(11):905-913.
15. Gao HJ, Liu PF, Li PW, Huang ZY, Yu FB, Lei T, Chen Y, Cheng Y, Mu QC, Huang HY: **Ligustrazine monomer against cerebral ischemia/reperfusion injury**. *Neural regeneration research* 2015, **10**(5):832-840.
16. Berdeaux A: **Nitric oxide: an ubiquitous messenger**. *Fundam Clin Pharmacol* 1993, **7**(8):401-411.
17. Chen HS, Chen X, Li WT, Shen JG: **Targeting RNS/caveolin-1/MMP signaling cascades to protect against cerebral ischemia-reperfusion injuries: potential application for drug discovery**. *Acta pharmacologica Sinica* 2018, **39**(5):669-682.
18. Huang Z, Huang PL, Ma J, Meng W, Ayata C, Fishman MC, Moskowitz MA: **Enlarged infarcts in endothelial nitric oxide synthase knockout mice are attenuated by nitro-L-arginine**. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism* 1996, **16**(5):981-987.
19. Zhao X, Haensel C, Araki E, Ross ME, Iadecola C: **Gene-dosing effect and persistence of reduction in ischemic brain injury in mice lacking inducible nitric oxide synthase**. *Brain research* 2000, **872**(1-2):215-218.
20. Ding D, Starke RM, Dumont AS, Owens GK, Hasan DM, Chalouhi N, Medel R, Lin CL: **Therapeutic implications of estrogen for cerebral vasospasm and delayed cerebral ischemia induced by aneurysmal subarachnoid hemorrhage**. *BioMed research international* 2014, **2014**:727428.
21. Brea D, Sobrino T, Ramos-Cabrer P, Castillo J: **Inflammatory and neuroimmunomodulatory changes in acute cerebral ischemia**. *Cerebrovascular diseases* 2009, **27 Suppl 1**:48-64.

Figures

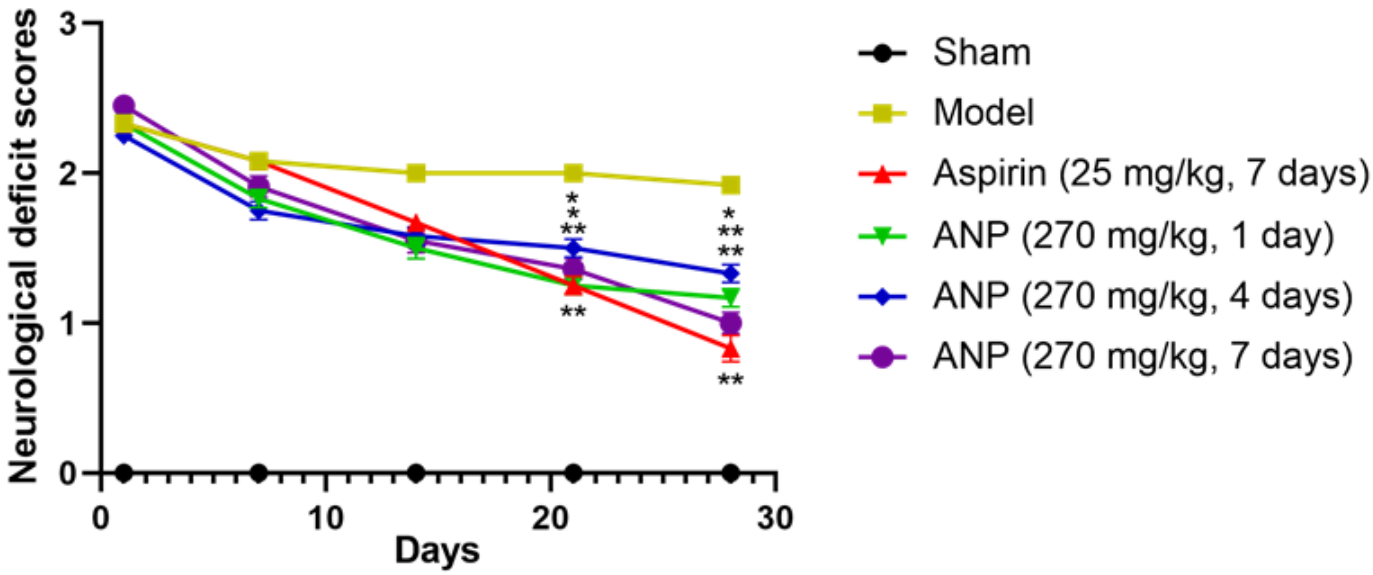

Figure 1

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.

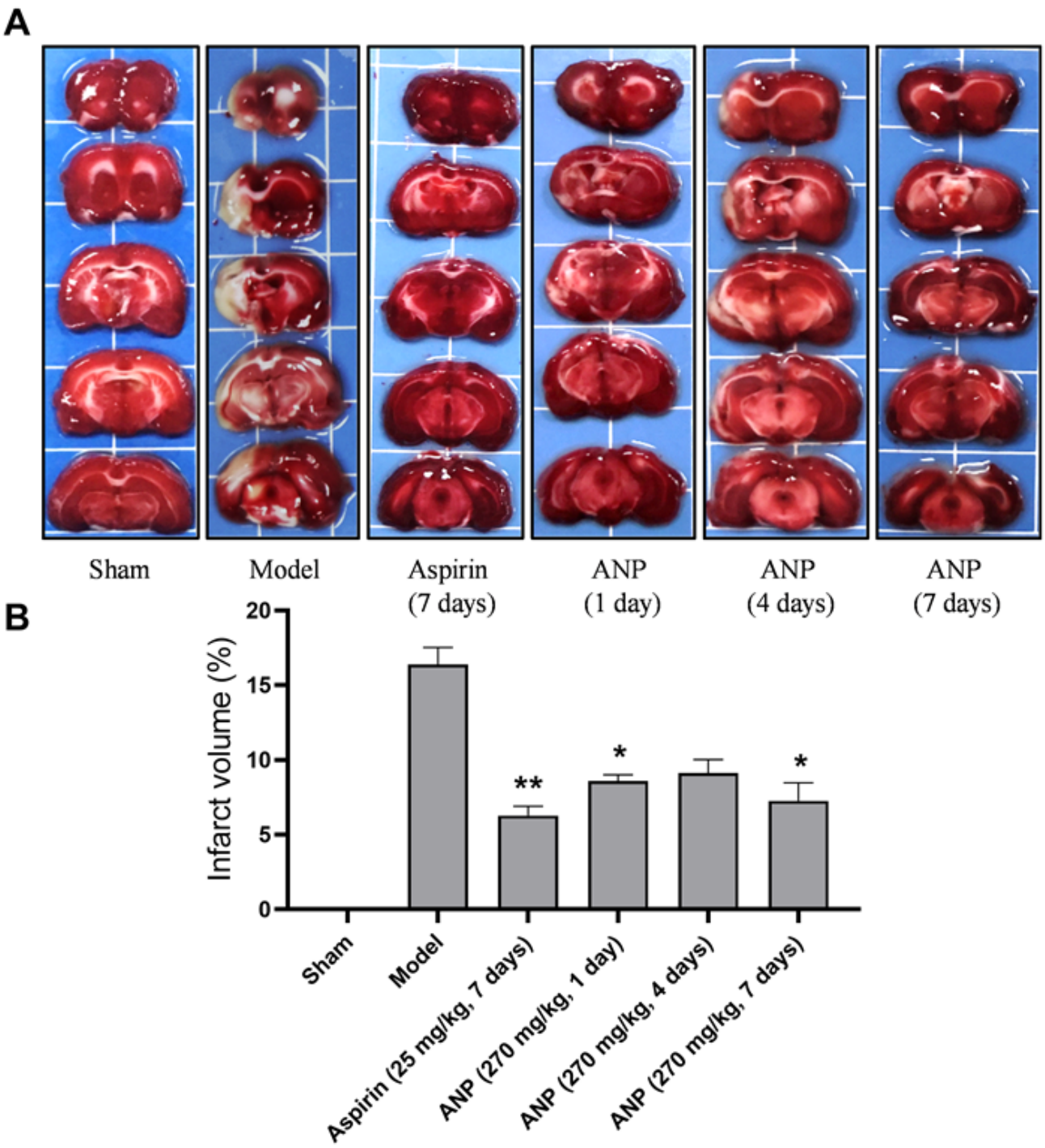

Figure 2

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.

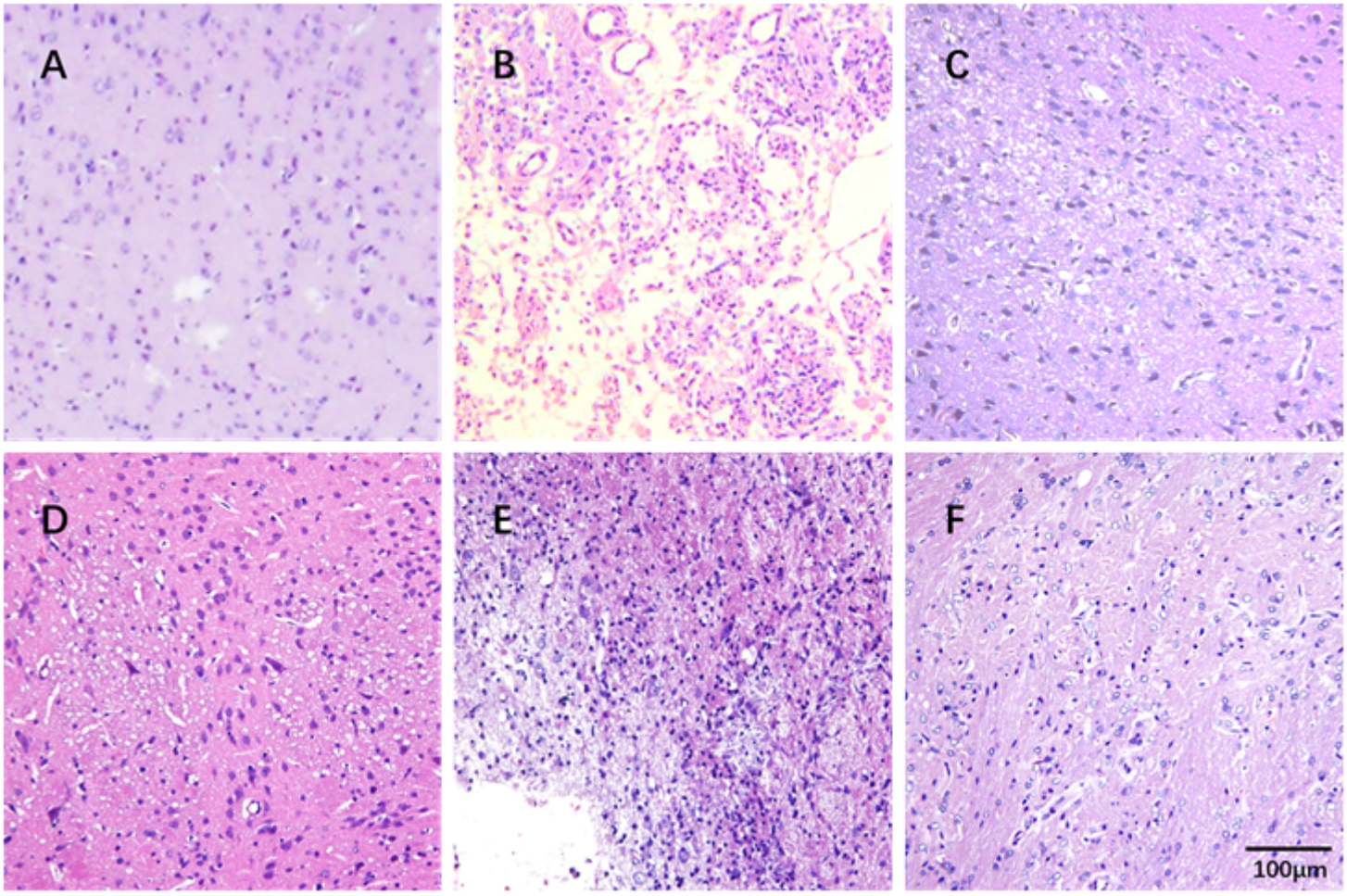

Figure 3

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).

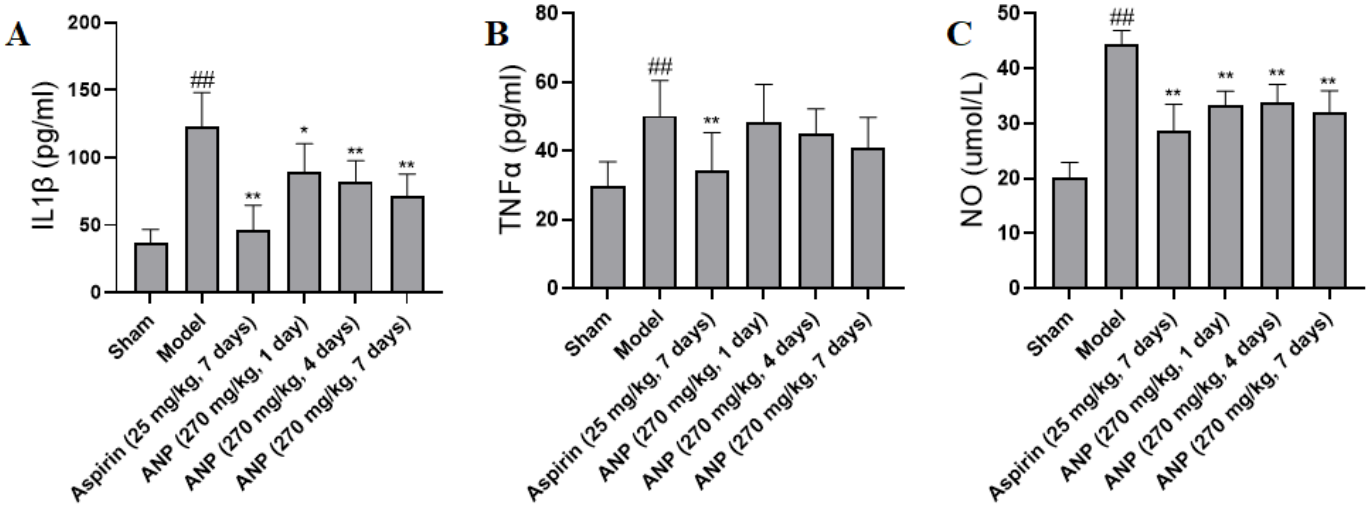

Figure 4

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).

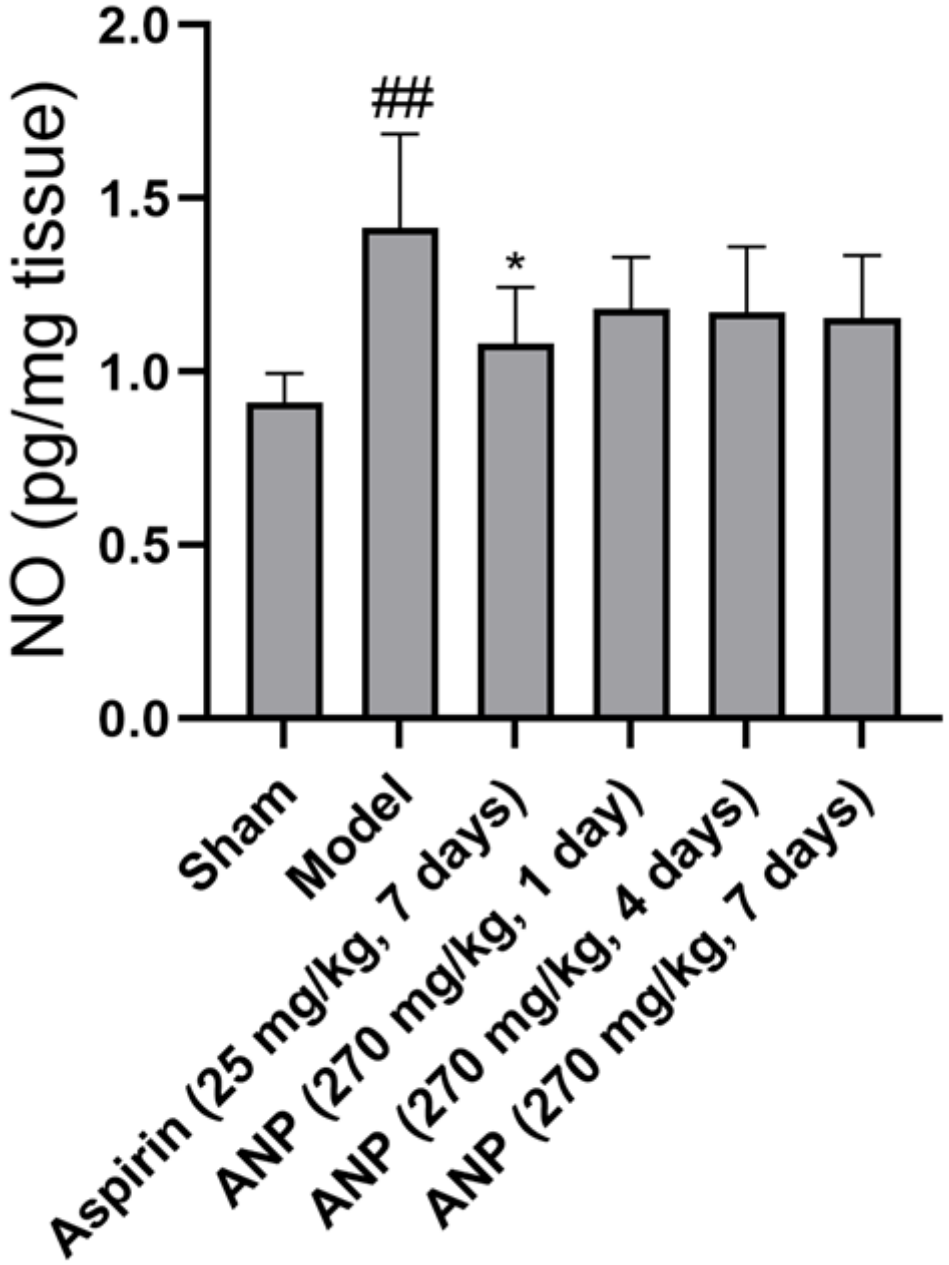

Figure 7

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.

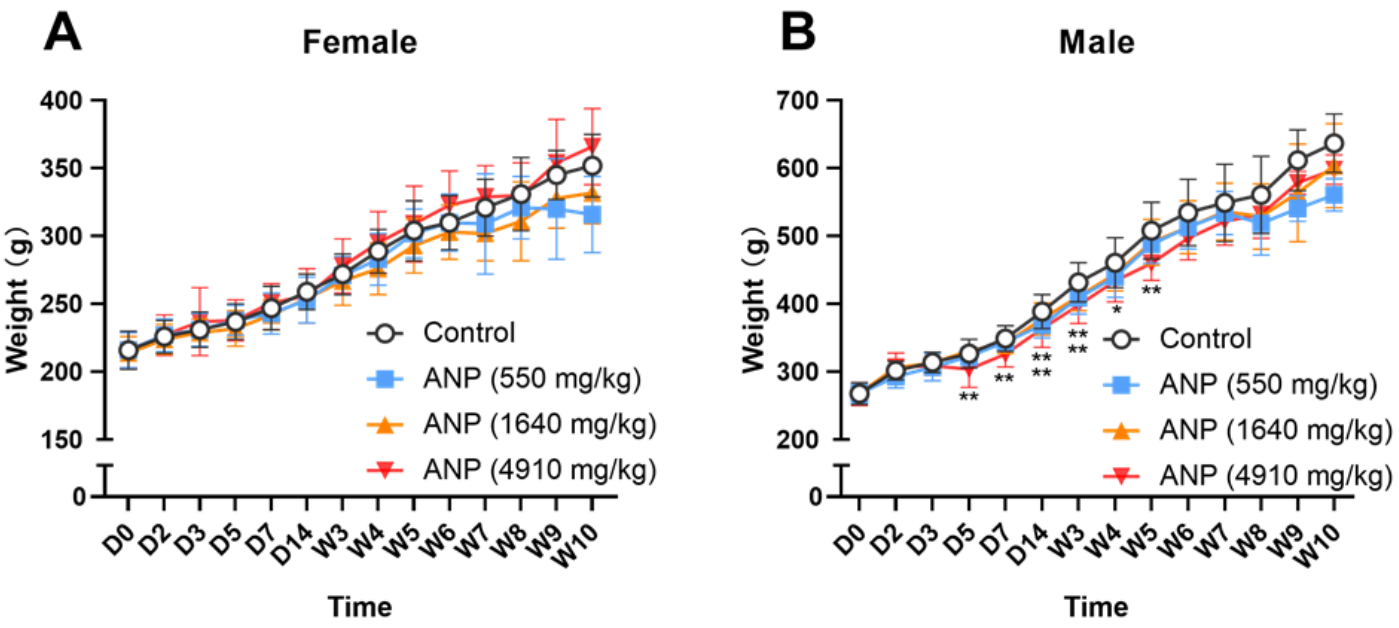

Figure 8

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