Chinese herbal medicine Naoxintong capsule combined with dual antiplatelet therapy in a rat model of coronary microembolization induced by homologous microthrombi

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Objective: In the present study, the efficacy of Naoxintong capsule (NXT), a compound Chinese herbal medicine, combined with dual antiplatelet therapy (DA) in a rat model of coronary microembolization (CME) was evaluated.

Methods: CME in rats was developed by injecting a suspension of microthrombotic particles into the left ventricle when the ascending aorta was obstructed. Microthrombotic particles were generated from the clots of rats sized by filtration through a screen (aperture diameter, 38 μm). A total of 95 rats were randomly divided into six groups, including control group, sham-operation (sham) group, CME model (CME) group, and NXT, DA, and NDA (NXT plus DA) groups. Rats in treatment groups were administrated intragastrically with NXT, DA, and NDA, respectively, from 3 d before to 7 d after operation. All rats were sacrificed on day 7 post-operationally, and samples of blood and heart were collected. The complete data of 69 rats were obtained. The incidence of CME (CME%) was evaluated by hematoxylin-eosin staining. Bleeding time (BT) and clotting time (CT) were measured by means of tail cutting and glass slide methods, respectively. Adenosine diphosphate-induced maximum platelet aggregation rate (PAR) was assessed with turbidimetry. Platelet counts were examined by an automated hematology analyzer. The levels of serum P-selectin, interleukin (IL)-6, IL-10, endothelin (ET-1) and endothelial nitric oxide synthase (eNOS) were all detected by enzyme-linked immunosorbent assay.

Results: Compared with control and sham groups, CME group had an increase in CME%, PAR, P-selectin, IL-6 and ET-1 (P<0.01, P<0.01), and a decrease in BT, CT, IL-10 and eNOS (P<0.01, P<0.01), compared with CME group, the groups receiving medications had an increase in BT, CT, IL-10 and eNOS (P<0.05 or P<0.01), and a decrease in CME%, PAR.

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P-selectin, IL-6 and ET-1 (P<0.05 or P<0.01), with DA group increasing most in BT and CT and decreasing most in PAR and P-selectin, and with NDA group increasing most in IL-10 and eNOS and decreasing most in CME%. IL-6 and ET-1. In terms of platelet counts, there was no statistically significant difference among groups (P>0.05).

**Conclusion:** NXT combined with DA can decrease CME%. The probable mechanism is that this therapy can appropriately inhibit platelet aggregation, balance the pro- and anti-inflammatory cytokines as well as serum ET-1 and eNOS. This therapy can also reduce risk of intraoperative bleeding during DA therapy.

**Keywords:** Naixinzong, Chinese patent drugs, coronary thrombosis, platelet aggregation inhibitors, chemokines, animal experimentation, rats

It was proved that drug-eluting stents (DES) was safe and effective in reducing the risk of restenosis after stenting\(^1,\)\(^2\), but often accompanied with late stent thrombosis associated with DES\(^3,\)\(^4\). It was reported a constant rate of late stent thrombosis of 0.6% per year for up to 3 and even 4 years after DES implantation\(^5,\)\(^6\). Studies such as CURE\(^7,\)\(^8\) and PCI-CURE\(^9,\)\(^10\) indicated that clopidogrel in addition to aspirin therapy was the basic treatment following percutaneous coronary intervention (PCI). Tavassoli et al\(^11\) reported that high maintenance dosage of clopidogrel was associated with a reduced risk of stent thrombosis. However, enhanced clopidogrel responsiveness is associated with a higher risk of nuisance bleeding complications\(^12,\)\(^13\).

Naixinzong capsule (NXT), a kind of Chinese patent medicine, is an approved drug for stroke\(^14\), which combined with aspirin could enhance the antiplatelet effect in patients with cardio-cerebrovascular diseases\(^15\). Therefore, we hypothesized that dual antiplatelet intervention with clopidogrel and aspirin (dual antiplatelet therapy, DA) combined with NXT (NDA) may have a better benefit to coronary microembolization (CME) in the present study.

**1 Materials and methods**

**1.1 Experimental methods**

**1.1.1 Animals** Adult male Sprague-Dawley (SD) rats weighing 280 to 320 g (animal license No. SCXK2007-0005) were obtained from the Animal Center, Fujian Medical University (Fuzhou, Fujian Province, China). The animals were handled according to the guidelines of the American Physiological Society\(^16\). Rats were housed in temperature (22±2°C) and (55±5)% humidity-controlled rooms with a 12-h on/12-h off light cycle. All the animals were allowed to move and take food with freedom. The Clinical and Animal Research Ethics Committees, Provincial Clinical College of Fujian Medical University approved all the procedures.

**1.1.2 Drugs and reagents** Naixinzong capsule (400 mg/capsule; main ingredients: Radix Astragali, Radix Paeoniae Rubra, Radix Salviae Miltiorrhizae, Radix Angelicae Sinensis, Rhizoma Chuanxiong, Semen Persicae, Flos Carthami, Resina Olibani, Myrrha, Caulis Spatholobi, Radix Achyranthis Bidentatae, Ramulus Cinnamomi, Ramulus Mori, Peretema, Scorpio, and Hirudo) was produced by Buchang Pharmaceutical Co., Ltd, China (drug approval number: ZZ20025001); clopidogrel bisulfate tablet (75 mg/tablet) was produced by Sanofi-Winthrop Industrie, France (batch No. J20080909); enteric-coated aspirin (25 mg/tablet) was produced by Shineway Pharmaceutical Co., Ltd, China (batch No. H13023716); benzylpenicillin (400 000 U/bottle) was produced by Tianjin Hualin Pharmaceutical Co., Ltd, China (batch No. H12020439); 10% chloral hydrate (200 mL/bottle) was prepared by Drug Preparation Room of Provincial Clinical College of Fujian Medical University; adenosine diphosphate (ADP) is produced by Sigma Co., Ltd, USA; rat P-selectin, interleukin-6 (IL-6), IL-10, endothelin (ET-1) and endothelial nitric oxide synthase (eNOS) enzyme-linked immunosorbent assay (ELISA) kits were all purchased from Sun Biomedical Technology Co., Ltd, Beijing, China.

**1.1.3 Instruments** Glass homogenizer (49KB-JPG, Sanhe Huaxing Glass Instrument Factory, Haimen, China); vortex agitator (XW80-A, Shanghai Medical University Instrument Factory, China); small animal ventilator (ALC-V8A, Shanghai Alcott Biotech Co., Ltd, China); optical microscope (CX41-12ec02, Olympus Company, Japan); Image-Pro Plus 5.0 image processing system (Media Cybernetics, Inc, USA); LBY-NJ4-channel platelet aggregation analyzer (Beijing Precil Instrument Co., Ltd, China); hematology analyzer (LH-750, Beckman Coulter Co., Ltd, USA).

**1.2 Experimental methods**

**1.2.1 Preparation of homologous microthrombotic particles** Thirty-six hours before establishment of the rat model, male SD rats weighing 280 to 320 g were sedated with 10% chloral hydrate at a dose of 3.0 mL/kg intraperitoneally. A total of 500 μL blood was withdrawn by puncturing the tail vein of rats and stored at room temperature for clot formation. The clot was fragmented by a glass homogenizer\(^17\), and the fragments were sized by filtration through a screen (aperture diameter, 38 μm), yielding microthrombi (microthromboemboli) with the size between 10 and 30 μm as measured by a micrometer. A total of 5 mg (about
1.5 × 10^{10} to 1.9 × 10^{10}) particles were suspended thoroughly in 0.3 mL calcium-free Dulbecco’s phosphate-buffered saline (PBS) on a vortex agitator prior to injection.

1.2.2 Establishment of the rat model Thirty-six hours later, the animals were reanesthetized with 10% chloral hydrate at a dose of 3.0 mL/kg intraperitoneally. Animals were ventilated with a small animal ventilator. A thoracotomy was performed at the middle line to ligate mammalian arteries, then the second and third intercostal spaces were cut open, then the pericardium was cut open and the ascending aorta was fully exposed. The microthrombi suspension in the PBS was injected as boluses with a 26-gauge needle into the left ventricular chamber during 10-s occlusion of the ascending aorta. The thoracic cavity and the skin incision were closed with sutures. For the age-matched sham-operation, the identical procedure was performed and PBS of the same volume was injected. After the operation, the rats were treated with benzylpenicillin (400 000 U for each rat) intraperitoneally for 3 d.

1.2.3 Experimental protocol and drug interventions In our preliminary experiment, we found that rats in DA group suffered higher incidence of death due to intraoperative bleeding. In order to ensure that each group had a certain number of survival rats, we used unequal randomization method as follows: 95 adult male SD rats were assigned with number 1 to number 95 according to the body weight from the lightest to the heaviest. The number 1 to number 95 rats got a 3-digit number in order from a 3-digit random number table, then we reordered the 95 3-digit numbers in order from the 1st to the 95th. Those rats matched the 1st to the 10th were assigned to control group; those matched the 11th to the 20th were assigned to sham-operation group; those matched the 21st to the 35th were assigned to CME group; those matched the 36th to the 50th were assigned to NDT group; those matched the 51st to the 80th were assigned to DA group; those matched the 81st to the 95th were assigned to NDA group. So there were control group (control, n = 10), sham-operation group (sham, n = 10), CME model group (CME, n = 15), and NXT (n = 15), DA (n = 30) and NDA (n = 15) treatment groups. The 75 rats in CME, NXT, DA and NDA groups underwent the procedures described before to create a CME model. Before CME modeling, rats in NXT, DA and NDA groups were pretreated with NDT suspension 2 mL/kg thrice daily (5 capsules dissolved in normal saline to produce 24 mL suspension, so every 2 mL suspension contained 166.67 mg NXT), DA (clopidogrel 7.81 mg/kg daily plus aspirin 7.81 mg/kg daily) and NDT + DA for 3 d, respectively. The drugs were intragastrically administered. The rat's dosage was defined as 6.25 folds of human's dosage (mg/kg body weight). After CME modeling, the above administrations were continued for 7 successive days (for rats in DA and NDA groups, clopidogrel 31.25 mg/kg daily was given for the first day after CME modeling). Those rats in sham and control groups were treated with equal amount (6 mL/(kg · d)) of normal saline. The complete data in 69 rats were obtained. All rats were sacrificed on day 7 post-operatively, and samples of blood and heart tissues were taken. The experimental protocol was schematically illustrated in Table 1. Among all the treatment groups, number of rats died from intraoperative bleeding in DA group was the most (P < 0.0083) during creation of CME model.

1.2.4 Evaluation of CME CME hearts were harvested, and fixed in 10% formalin, then embedded and sectioned into slices of 5 μm in thickness. The sections (trans-sections) obtained at the level of atrioventricular groove of the left ventricle were used for the following morphological assessment. The general morphology was evaluated by hematoxylin-eosin (HE) staining. The number of microemboli in the HE-stained left ventricular section was counted in 10 random fields (Light microscopy, ×100) from 100 random coronary arterioles (50 to 100 μm) in each section. The incidence of CME (CME%) was expressed by the percent of coronary arterioles occupied by microemboli in this 100 random coronary arterioles (50 to 100 μm),

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Pretreatment (3 d before experiment)</th>
<th>Model</th>
<th>Accidental death (n)</th>
<th>Death due to intraoperative bleeding (n)</th>
<th>Death after operation (n)</th>
<th>Survival (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>No treatment</td>
<td>Sham-operation; normal saline 6 mL/(kg · d)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Sham</td>
<td>10</td>
<td>Normal saline 6 mL/(kg · d)</td>
<td>CME, normal saline 6 mL/(kg · d)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>CME</td>
<td>15</td>
<td>Normal saline 6 mL/(kg · d)</td>
<td>CME, normal saline 6 mL/(kg · d)</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>11</td>
</tr>
<tr>
<td>NXT</td>
<td>15</td>
<td>NXT 500 mg/(kg · d)</td>
<td>CME, NXT 500 mg/(kg · d)</td>
<td>1</td>
<td>0</td>
<td>3</td>
<td>11</td>
</tr>
<tr>
<td>DA</td>
<td>30</td>
<td>DA 7.81 mg/(kg · d)</td>
<td>CME, DA 7.81 mg/(kg · d)</td>
<td>1</td>
<td>13</td>
<td>3</td>
<td>13</td>
</tr>
<tr>
<td>NDA</td>
<td>15</td>
<td>NXT 500 mg/(kg · d) plus DA 7.81 mg/(kg · d)</td>
<td>CME, NXT 500 mg/(kg · d)</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>14</td>
</tr>
<tr>
<td>Total</td>
<td>95</td>
<td></td>
<td>—</td>
<td>2</td>
<td>13</td>
<td>11</td>
<td>69</td>
</tr>
</tbody>
</table>

Sham; sham-operation; CME, coronary microembolization; DA, dual antiplatelet therapy; NDA, Naoxintong capsule plus DA.
i.e. CME% = (total number of microemboli of 100 random coronary arterioles (50 to 100 μm)/100) × 100%. Then we got the average CME% of the 10 random fields, which was the CME% of this section. A detailed microscopic examination for the presence of CME was defined by two pathologists. All morphological quantification was performed by observing the whole section under the assistance of an Image-Pro Plus 5.0 image processing system. As shown in Figure 1, the microemboli were characterized by platelet aggregation, fibrin (as the arrow pointed in Figure 1) and other blood particles.

![Figure 1 Microemboli in coronary arterioles](image)

**Figure 1**: Microemboli in coronary arterioles (50 to 100 μm) of coronary microembolization model rats. Hematoxylin-eosin staining (Light microscopy, ×400); bar in the photograph represents 20 μm.

1.2.5 Determination of blood parameters On the 7th day after operation, bleeding time (BT) and clotting time (CT) were measured by means of tail cutting and glass slide methods before animals were sacrificed.

One part of blood was collected from the abdominal aorta by using a vacuum tube containing 3.8% citrate plasma on the 7th day after operation, then adenosine diphosphate (ADP)-induced maximum platelet aggregation rate (PAR) was achieved. In brief, samples were centrifuged at 700 r/min for 10 min to obtain platelet-rich plasma (PRP) and additionally 10 min at 3,500 r/min to obtain platelet-poor plasma (PPP). Percent of platelet aggregation after being stimulated by 5 μmol/L and 20 μmol/L ADP respectively were assessed with turbidimetry by using a LBY-NJ4-channel platelet aggregation analyzer. A Beckman Coulter LH-750 hematology analyzer was used to examine platelet counts.

Another part of blood from the abdominal aorta was collected by using a vacuum tube containing sodium heparin on the 7th day after operation. Samples were centrifuged at 2,000 r/min for 15 min to obtain plasma, then stored in a −80°C freezer for subsequent ELISA analyses. Serum levels of P-selectin, IL-6, IL-10, ET-1 and eNOS were stringently determined by using ELISA kits according to reagent instructions, respectively.

1.3 Statistical analysis The data were analyzed by using the SPSS 16.0 for Windows. All values were presented as $\bar{x} \pm s$. The enumeration data analysis was conducted by chi-square test; comparisons among different sample rates were conducted by partition of chi-square; the measurement data analysis were conducted by one-way analysis of variance, followed by SNK-q test for homogeneity of variance and by Game-Howell test for heterogeneity of variance; correlation analysis between two variables was conducted by Spearman rank correlation analysis. $P < 0.05$ was considered to be statistically significant.

2 Results

2.1 Pathohistological findings There were morphological findings detected by HE staining. CME% values were negligible in control and sham groups, but markedly increased in CME group ($P < 0.01$). In comparison with untreated CME group, the groups receiving medications had a decrease in CME%, with NDA group decreasing most obviously ($P < 0.01$). See Figure 2 and Table 2. The results indicated that drug intervention could decrease CME% in model rats and NDA therapy had the best antithrombosis effect.

![Figure 2 Morphological changes of CME% in different groups](image)

**Figure 2**: Morphological changes of CME% in different groups. The morphological changes were detected by HE staining (Light microscopy, ×100). A: Control group; B: Sham-operation group; C: Coronary microembolization model group; D: Naxintong capsule group; E: Dual antiplatelet therapy group; F: Naxintong capsule plus dual antiplatelet therapy group. Bars in the photographs represent 20 μm.
Table 2 Comparison of CME% in different groups after drug interventions

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>CME%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>0.90±0.15**</td>
</tr>
<tr>
<td>Sham</td>
<td>10</td>
<td>0.12±0.14**</td>
</tr>
<tr>
<td>CME</td>
<td>11</td>
<td>29.04±1.85</td>
</tr>
<tr>
<td>NXT</td>
<td>11</td>
<td>25.65±1.92**</td>
</tr>
<tr>
<td>DA</td>
<td>13</td>
<td>21.03±1.82**</td>
</tr>
<tr>
<td>NDA</td>
<td>14</td>
<td>18.80±1.97**</td>
</tr>
</tbody>
</table>

** P<0.01, vs CME group; ▲▲ P<0.01, vs NXT group; ▲▲▲ P<0.01, vs DA group. Sham: sham-operation; CME: coronary microembolization; NXT: Naoxintong capsule; DA: dual antiplatelet therapy; NDA: Naoxintong capsule plus DA; CME%: incidence of CME.

2.2 Changes of blood parameters
2.2.1 BT and CT  BT and CT of rats after drug interventions in each group were showed in Table 3. Compared with control and sham groups, CME group had a decrease in BT and CT (P<0.01, P<0.01). Compared with untreated CME group, the groups receiving medications had an increase in BT and CT, with DA group increasing most obviously (P<0.05 or P<0.01). The longest BT and CT in DA group could explain why rats in DA group suffered from the highest rate of death due to intraoperative bleeding during CME modeling.

2.2.2 PAR and platelet counts  Comparisons of PAR and platelet counts among groups were shown in Table 4. In terms of platelet counts, there was no statistically significant difference among groups (P>0.05). Compared with control and sham groups, CME group had an increase in PAR (P<0.01, P<0.01). Compared with untreated CME group, the groups receiving medications had a decrease in PAR (P<0.01), with DA group decreasing most obviously. The results indicated that the activation of platelet was inhibited after drug treatment and DA therapy had the strongest inhibition effect on platelet aggregation.

2.3 P-selectin, IL-6, IL-10, ET-1 and eNOS  Comparisons of P-selectin, IL-6, IL-10, ET-1 and eNOS among groups were shown in Table 5. Compared with control and sham groups, CME group had an increase in P-selectin, IL-6 and ET-1, and a decrease in IL-10 and eNOS (P<0.01, P<0.01). Compared with untreated CME group, the groups receiving medications had increases in IL-10 and eNOS (P<0.05 or P<0.01), and decreases in P-selectin, IL-6 and ET-1 (P<0.01), with DA group decreasing most in P-selectin, and with NDA group increasing most in IL-10 and eNOS and decreasing most in IL-6 and ET-1. The results demonstrated that NDA therapy had the best effect in regulating pro- and anti-inflammatory cytokines and regulating serum ET-1 and eNOS among the therapies used in this study.

Table 3 Comparisons of BT and CT among groups

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>BT (min)</th>
<th>CT (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>6.05±1.20**</td>
<td>177.60±24.27**</td>
</tr>
<tr>
<td>Sham</td>
<td>10</td>
<td>5.92±1.10**</td>
<td>174.20±23.20**</td>
</tr>
<tr>
<td>CME</td>
<td>11</td>
<td>4.59±1.56</td>
<td>141.91±26.07</td>
</tr>
<tr>
<td>NXT</td>
<td>11</td>
<td>23.47±3.62*</td>
<td>253.09±35.43*</td>
</tr>
<tr>
<td>DA</td>
<td>13</td>
<td>42.43±3.58**</td>
<td>322.08±31.33**</td>
</tr>
<tr>
<td>NDA</td>
<td>14</td>
<td>39.93±3.01**</td>
<td>291.07±29.66**</td>
</tr>
</tbody>
</table>

** P<0.01, vs CME group; ▲ P<0.01, vs NXT group; ▲▲ P<0.01, vs DA group. Sham: sham-operation; CME: coronary microembolization; NXT: Naoxintong capsule; DA: dual antiplatelet therapy; NDA: Naoxintong capsule plus DA; BT: bleeding time; CT: clotting time.

Table 4 Comparisons of PAR and platelet count among groups

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>PAR to 5 μmol/L ADP (%)</th>
<th>PAR to 20 μmol/L ADP (%)</th>
<th>Platelet count (X10^9/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>49.35±6.51**</td>
<td>53.65±3.43**</td>
<td>1146.70±179.17</td>
</tr>
<tr>
<td>Sham</td>
<td>10</td>
<td>52.14±3.73**</td>
<td>55.75±6.47**</td>
<td>1176.00±197.90</td>
</tr>
<tr>
<td>CME</td>
<td>11</td>
<td>63.61±4.67</td>
<td>68.50±5.73</td>
<td>1202.00±187.37</td>
</tr>
<tr>
<td>NXT</td>
<td>11</td>
<td>39.00±5.43**</td>
<td>44.41±6.95**</td>
<td>1182.10±172.49</td>
</tr>
<tr>
<td>DA</td>
<td>13</td>
<td>5.25±3.79**</td>
<td>10.63±5.62**</td>
<td>1184.90±156.79</td>
</tr>
<tr>
<td>NDA</td>
<td>14</td>
<td>8.53±4.69**</td>
<td>24.89±7.56**</td>
<td>1181.10±147.51</td>
</tr>
</tbody>
</table>

** P<0.01, vs CME group; ▲▲ P<0.01, vs NXT group; ▲▲▲ P<0.01, vs DA group. Sham: sham-operation; CME: coronary microembolization; NXT: Naoxintong capsule; DA: dual antiplatelet therapy; NDA: Naoxintong capsule plus DA; PAR: platelet aggregation rate; ADP: adenosine diphosphate.
Table 5 Comparison of serum P-selectin, IL-6, IL-10, ET-1 and eNOS among groups

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>P-selectin (μg/L)</th>
<th>IL-6 (ng/L)</th>
<th>IL-10 (ng/L)</th>
<th>ET-1 (ng/L)</th>
<th>eNOS (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>8.80±0.34**</td>
<td>84.05±1.09**</td>
<td>11.95±0.56**</td>
<td>0.93±0.05**</td>
<td>11.99±0.54**</td>
</tr>
<tr>
<td>Sham</td>
<td>10</td>
<td>13.85±0.55**</td>
<td>90.92±3.68**</td>
<td>19.54±1.72**</td>
<td>0.98±0.05**</td>
<td>12.25±0.75**</td>
</tr>
<tr>
<td>CME</td>
<td>11</td>
<td>23.06±0.88</td>
<td>905.00±45.71</td>
<td>5.32±0.32</td>
<td>2.93±0.11</td>
<td>5.60±0.30</td>
</tr>
<tr>
<td>NXT</td>
<td>11</td>
<td>5.94±0.10</td>
<td>451.59±16.75</td>
<td>18.59±0.74**</td>
<td>2.20±0.19**</td>
<td>25.00±1.37**</td>
</tr>
<tr>
<td>DA</td>
<td>13</td>
<td>2.24±0.10</td>
<td>247.01±8.66**</td>
<td>8.37±0.77**</td>
<td>1.92±0.04**</td>
<td>21.27±0.79**</td>
</tr>
<tr>
<td>NDA</td>
<td>14</td>
<td>3.89±0.10</td>
<td>158.49±6.71**</td>
<td>12.24±1.18**</td>
<td>1.52±0.04**</td>
<td>43.68±2.32**</td>
</tr>
</tbody>
</table>

* P<0.05, ** P<0.01, vs CME group; △△ P<0.01, vs NXT group; △△△ P<0.01, vs DA group. Sham: sham-operation; CME: coronary microembolization; NXT: Naxizong capsule; DA: dual antiplatelet therapy; NDA: Naxizong capsule plus DA; IL: interleukin; ET-1: endothelin-1; eNOS: endothelial nitric oxide synthase.

2.3 Correlation analysis
Correlations between IL-6 and IL-10, ET-1 and eNOS, and between P-selectin and PAR to 5 μmol/L ADP (%) or 20 μmol/L ADP (%) were analyzed. The strongest correlations existed between P-selectin and PAR to 5 μmol/L ADP (%) (r = 0.829, P<0.001) and P-selectin and PAR to 20 μmol/L ADP (%) (r = 0.821, P<0.001). More modest but significant correlation existed between IL-6 and IL-10 (r = -0.492, P<0.001). There was no significant correlation between ET-1 and eNOS (r = -0.168, P=0.168).

3 Discussion
Atherosclerotic plaque rupture, occurring either spontaneously or during coronary interventions or during the procedure of thrombolytic, results in the release of atheromatous and (or) thrombotic material into the coronary circulation, which may embolize the microvascular bed, leading to CME[17, 18]. The incidence of CME is difficult to judge. Spontaneous CME occurs, but most likely, only the tip of the iceberg could be recognized. Periprocedural CME occurs on average in 25% of all patients undergoing PCI, but its incidence ranges from 0% to 70%, in part depending on the method of its assessment[19]. Thus, reducing the incidence of CME has become a big attention in cardiovascular field. Unfortunately, there are only few measures to completely prevent or effectively treat CME until now, albeit several treatments such as thrombus aspiration/filtration devices and drug therapies (adenosine, nitroglycerine, diltiazem, glycoprotein Ilb/Ilia antagonist, statins etc.) have been used in clinical practices[20-26]. However, these measures have their limitations, such as antiplatelet drug resistance[27], which is a big problem. What is more, antiplatelet drugs frequently lead to imbalance between blood coagulation system and anticoagulant system. This was characterized by nuisance bleeding implications[10, 11]. Therefore, new therapeutic options need to be investigated.

For further CME studies and new therapeutic options-related researches, developing an animal model more close to the clinical real world of CME is of importance. In our present study, a CME model in rats was successfully created by using homologous microthrombotic particles, produced by homologous blood clot which was rich in platelet, fibrin and other blood particles. These homologous microthrombotic particles could more closely mimic the components of clinical coronary emboli and better simulate CME as occurring in clinical scenarios[16]. Therefore, we attempted to develop a reproducible CME model with homologous microthrombi in rats in our study.

There has been an emerging trend of multitarget drug development, as well as an increasing interest in traditional Chinese medicine (TCM) that applies a more holistic treatment to complex and multifactorial diseases. The physiopathologic changes presented in the atherosclerosis, including CME-forming process, such as thrombus formation, vascular wall inflammation, cell proliferation and matrix accumulation, are recognized by TCM as internally associated with blood stasis syndrome[28]. Thousands of years of clinical practices in TCM have accumulated a number of formulas that exhibit reliable efficacy and safety in vivo, and the Chinese drugs for activating blood circulation and removing stasis are proved to be capable of antagonizing platelet aggregation, adjusting lipid metabolism, inhibiting smooth muscular cell proliferation, etc. Buyang Huanwu Decoction (BYHWD), a classic formula for activating blood circulation and removing stasis, has been recognized as a treatment for coronary heart disease with qi deficiency and blood stasis syndrome and cerebrovascular disease in clinical practices. NXT, a Chinese patent medicine of BYHWD, is an approved drug for stroke[12], which combined with aspirin could enhance the antiplatelet effect in patients with cardio-cerebrovascular diseases[13]. Among the components of NXT, high dosage of Huangqi (Radix Astragali) as the monarch drug could tonify qi and invigorate blood circulation, in another word, it can enhance the patient’s immunity, dilate coronary vasculature, improve the conditions of myocardial ischemia and hypoxia; Shuizhi (Hirudo), Quanxie (Scorpion) and Dilong (Pheretima) as ministerial drugs contain a large number of thrombolytic factors, which can inhibit platelet aggregation and thrombus formation, significantly reduce blood viscosity and improve tissue circula-
tion; Taoren (Semen Persicae), Honghua (Flos Carthami), Ruxiang (Olibanum), Moyao (Myrrha), Chuanxiong (Rhiizaoma Chuanxiong), Chishao (Radix Paeoniae Rubra), Danshen (Radix Salviae Miltiorrhizae) and Jixueteng (Caulis Spatholobi) can activate blood circulation and remove stasis; Danggui (Radix Angelicae Sinensis), Sangzhi (Ramulus Mori), and Guizhi (Ramulus Cinnamomi) can enhance the efficacy of activating blood circulation and removing stasis; all these herbs enable NXT to be efficacious in activating blood circulation and removing stasis and strongly improve microcirculation.

Previous studies showed that platelet activation plays a fundamental role in CME. Davies et al.\textsuperscript{[21]} reported that platelet aggregation was found distal to an atherosclerotic epicardial coronary plaque that had developed fissuring and mural thrombosis in 30% of 90 patients who died suddenly of ischemic heart disease. Platelets that adhere to the vessel wall at sites of endothelial cell activation contribute to the development of chronic atherosclerotic lesions, and when these lesions rupture, they trigger the acute onset of arterial thrombosis. This suggests that platelet activation increases the possibility of plaque formation\textsuperscript{[20]}. Activated platelets can influence the progression of plaque formation by releasing adhesive ligands, such as P-selectin, that become expressed on the platelet membrane and mediate platelet-endothelium interactions\textsuperscript{[30]}. Our data demonstrated the strongest correlation between P-selectin and PAR to 5 \( \mu \text{mol/L} \) ADP (\( r = 0.829, P < 0.001 \)) and between P-selectin and PAR to 20 \( \mu \text{mol/L} \) ADP (\( r = 0.821, P < 0.001 \)). This was consistent with the results of previous studies. Platelet aggregation is considered to be a major causal component in arterial thrombosis. Inhibition of platelet aggregation leads to the acute and chronic protection of patients with atherothrombotic disease\textsuperscript{[31]}. Therefore, suppressing platelet aggregation can decrease the possibility of microthrombus formation with subsequent decrease of CME%.

Our results showed that, in comparison with untreated CME group, the groups receiving medications had a decrease in PAR, with DA group decreasing most obviously (\( P < 0.01 \)), which indicated that platelet activation was inhibited after drug treatment and DA therapy had the strongest inhibition to platelet aggregation. This may be a major mechanism that rats in DA group suffered from the highest rate of death due to intraoperative bleeding during CME modeling and had the longest BT and CT. However, rats in NDA group did not have death due to intraoperative bleeding during CME modeling and had moderate BT and CT, and more importantly, with the least CME%. This “contradiction” indicates that platelet activation is not the only mechanism of CME, however, other mechanisms may involve in the formation and development of CME.

The results also suggested that NDA might provide more appropriate inhibition of platelet-mediated thrombosis. Generally, the enhancement of antiplatelet benefit is frequently associated with an increase in bleeding risk. This may be due to the over-inhibition of the thromboxane A\(_2\) and ADP platelet activation pathways. Fortunately, this benefit may not be accompanied by increased risk of bleeding when NDA therapy was used. This bidirectional regulation keeps the balance between anti-CME efficacy and bleeding risk of antiplatelet therapy. These facts suggested that Chinese drug NXT might provide a multitarget therapy and bidirectional regulative effect of regulating the yin and yang balance\textsuperscript{[22]}, and they might act, on the one hand, to decrease CME%, and on the other hand, to reduce bleeding risk.

Cytokine-mediated inflammation accompanies atherosclerosis from its initiation to the occurrence of clinical endpoints\textsuperscript{[23]}. IL-6, in particular, a potent inflammatory cytokine, is produced in a variety of tissues, including activated leucocytes, adipocytes and endothelial cells, and influences diverse cellular actions, including effects on platelets, endothelium, factors of metabolism and coagulation\textsuperscript{[24, 25]}. Moreover, IL-6 has been recognized as the best predictor of critical coronary stenosis with the highest sensitivity and specificity\textsuperscript{[26]}. Conversely, the anti-inflammatory cytokine IL-10 seems to have an athero-protective role\textsuperscript{[36]}. IL-10 has multifaceted anti-inflammatory properties, and is capable of down-regulating numerous inflammatory pathways, thus plays an important role in the progression and instability of the atherosclerotic plaque\textsuperscript{[37]}. IL-10 has also been shown to impair atherosclerosis in various animal models and has been proposed as an “immunological scalpel” in the atherosclerotic process\textsuperscript{[38]}. In the present study, we showed that, in comparison with untreated CME group, the groups receiving medications had an increase in IL-10 and a decrease in IL-6, with NDA group increasing most in IL-10 and decreasing most in IL-6 (\( P < 0.05 \) or \( P < 0.01 \)). These results demonstrated that NDA was more efficacious in anti-inflammatory therapies than DA or NXT. There are data showing that inflammation and thrombosis are closely linked\textsuperscript{[39]}. Inflammation can beget local thrombosis and thrombosis can amplify inflammation, so inhibiting inflammation can suppress thrombosis, which may be one of the underlying mechanisms that NDA has the best antithrombosis effect, with the least CME% in NDA group. Our data also demonstrated modest but significant negative correlation between IL-6 and IL-10 (\( r = -0.492, P < 0.001 \)). Overall, the data of the present study now support that NDA therapy can best regulate pro- and anti-inflammatory cytokines, partly due
to high dosage of Huangqi (Radix Astragali, monarch drug of NXT), which can tonify qi, in another word, enhance human body’s immunity, as a result of inhibiting inflammation response and may finally stabilize atherosclerotic plaques and decrease periprocedural CME.

It is known that vascular endothelium cells provide potent anticoagulant properties under physiological conditions, preventing both the initiation and propagation of the coagulation process. However, endothelial cells can rapidly shift the hemostatic balance from an antithrombotic to a prothrombotic state when endothelial cells are injured. This may be caused by increased binding and activity of coagulation factors, loss of anticoagulant surface components, and prothrombotic substances, such as tissue factor, factor V, platelet activating factor, von Willebrand factor, and plasminogen activator inhibitor (P-3). Vascular endothelial cells exclusively produce ET-1. This may contribute to microvasculature dysfunction due to its potent vasoconstrictive property. Elevated ET-1 level has been reported to be an independent predictor of a more rapid progression of coronary artery atherosclerosis as measured by coronary angiography. Nitric oxide (NO) is known to be the most important endothelium-derived substance that regulates platelet function, vasomotor tone as well as blood flow and inhibits neutrophil adhesion to the endothelium. NO is generated by a class of enzymes known as NO synthases (NOS). Three main isoforms of NOS have so far been described, the constitutive isoforms neuronal NOS (nNOS) and endothelial NOS (eNOS) and the inducible isoform (iNOS). It has been reported that inhibitory effects on eNOS-derived NO may lead to enhanced platelet activation, thrombosis and endothelial dysfunction, i.e., impaired endothelium-derived vascular relaxation. Overall, both high plasma ET-1 levels and low plasma eNOS levels (with subsequent low eNOS-derived NO) are markers of endothelial cell injury. Our results showed that compared with control and sham groups, CME group had an increase in ET-1 and a decrease in eNOS (\(P < 0.01\), \(P < 0.01\)), which is indicative of vascular endothelial dysfunction in untreated CME model rats; in comparison with untreated CME group, the groups receiving medications had an increase in eNOS and a decrease in ET-1, with NDA group increasing most in eNOS and decreasing most in ET-1 (\(P < 0.01\), \(P < 0.01\)), indicating that NDA therapy had the best efficacy in regulating serum ET-1 and eNOS levels, thus resulting in markedly improved vascular endothelial function. Because vascular endothelium cells provide potent anticoagulant properties which inhibit thrombosis, NDA therapy can be the best way of inhibiting microthrombosis. This may be another complex mechanism that NDA therapy has the best anti-thrombosis effect, with the least CME% in NDA group.

In summary, our results confirmed that NXT combined with DA could decrease CME%, which may be mainly due to the mechanisms of appropriately inhibiting platelet aggregation, regulating pro- and anti-inflammatory cytokines and regulating serum ET-1 and eNOS, and thus reducing the bleeding risk of DA. However, the precise mechanisms still need further studies, especially, the mechanism that NDA could reduce bleeding risk. It is known that there are differences between murine and human hemostatic systems, so can human benefit from the safety and efficacy of NDA therapy which have been proved in CME model rats in our study? These are issues waiting for further studies.

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脑心通胶囊联合双联抗血小板疗法防治
大鼠自体微血栓所致冠状动脉微栓塞

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目的：探讨脑心通胶囊（Naoxtiong capsule，NXT）联合双联抗血小板疗法（dual antiplatelet therapy，DA）对大鼠自体冠状动脉微栓塞（coronary microembolization，CME）的治疗效果。

方法：在关闭升主动脉的同时自心尖部注入同源大鼠微血栓（大鼠自体血凝块经 38 μm 的滤网过滤制成）建立 CME 模型。95 只大鼠不均等随机分成 6 组，即空白组（n=10）、假手术组（n=10）、CME 组（n=15）、NXT 组（n=15）、DA 组（n=30）、NXT联合 DA 组（NDA 组，n=15）。各组术前预给药 3 d，术后继续用药 7 d，取血后处死动物收集标本。共计 69 只大鼠获得完整数据。苏木精-伊红染色观察 CME 数量百分比（CME%），剪尾法检测出血时间（bleeding time，BT），玻片法检测凝血时间（clotting time，CT），比浊法检测二磷酸腺苷诱导的最大血小板聚集率（platelet aggregation rate，PAR），自动血液分析仪检测血小板计
数，酶联免疫吸附试验法检测 P 选择素、白细胞介素 6（interleukin-6，IL-6）、IL-10、内皮素 1（endothelin-1，ET-1）及内皮型一氧化氮合酶（endothelial nitric oxide synthase，eNOS）。

结果：与空白组及假手术组比较，CME 组的 CME%、PAR、P 选择素、IL-6 和 ET-1 明显升高（P<0.01），BT、CT、IL-10 和 eNOS 明显降低（P<0.01）。与 CME 组比较，各药物处理组的 BT、CT、IL-10 和 eNOS 明显升高（P<0.05 或 P<0.01），CME%、PAR、P 选择素、IL-6 和 ET-1 明显降低（P<0.05 或 P<0.01）。其中 DA 组的 BT 和 CT 最长，PAR 和 P 选择素最低，而 NDA 组的 IL-10 和 eNOS 最高，CME%、IL-6 和 ET-1 最低；血小板计数在各组中无统计学差异（P>0.05）。

结论：脑脊液通路联合双联抗血小板治疗可能通过抑制血小板聚集、调节促炎因子与抗炎因子以及 ET-1 与 eNOS 的平衡显著减少 CME 模型大鼠的 CME%。脑脊液通路联合双联抗血小板治疗还能够减少双联抗血小板治疗出血的危险性。

关键词：脑脊液通路；成药药；冠状动脉血栓形成；血小板聚集抑制剂；炎症趋化因子；动物实验；大鼠

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