Evaluation of the anti-inflammatory and antioxidant pharmacodynamic components of naoxintong capsules as a basis of broad spectrum effects

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Abstract

Context: Naoxintong capsule (NXT) is one of the most prevalent Traditional Chinese Medicine formulations in the treatment of coronary heart disease (CHD), yet the action of pharmacodynamic components remains unclear.

Objective: To determine the basis by which pharmacodynamic components of NXT may be effective in the treatment of CHD.

Materials and methods: The protective effect of NXT (0.01–100 μg/mL) on 293 T and hy926 cells was determined by MTT assay for 24 h. Afterwards, to investigate the pharmacodynamic material basis of NXT in anti-inflammatory and antioxidant effects, based on previous UPLC/Q-TOF analysis, 293 T and hy926 cells were divided into control (treated with solvent), model (incubated with TNF-α, LPS or H2O2), intervention (treated with UPLC components) and positive groups. After 24 h of treatment, all cells were tested to verify the screening results. MOE software was applied to dock bioactive compounds with phosphoinositide 3-kinase (PI3K), then the protein expression and phosphate levels were determined by western blotting.

Results: NXT could significantly inhibit the expression of NF-kB, MMP-9 and NO in cells with IC50 values of 0.1178, 0.1182 and 0.1094 μg/mL. Based on the screening results, six components of NXT were identified (calycosin, ferulic acid, salvianolic acid B, ononin, salvianolic acid E, and salvianolic acid F) which can inhibit NF-kB, MMP-9, and NO simultaneously, while exerting cytoprotective effects by inhibiting the activation of the PI3K/AKT pathway under different conditions by virtue of their advantageous interaction with PI3K.

Conclusions: These ingredients have outstanding therapeutic potential and may provide a scientific basis for the future application and research of NXT.

Introduction

Coronary heart disease (CHD) has high morbidity and mortality, and is one of the most serious diseases that has aroused widespread concern globally (Anderson et al. 2016). In China, CHD is one of the leading causes of death (Ma et al. 2016a). CHD is a chronic inflammatory disorder; plaque formation can cause the narrowing of the coronary artery, the blood supply to the heart is then reduced, thereby triggering CHD (Chhibber-Goel et al. 2016). It is well established that atheromatous, dyslipidemia, and endothelial dysfunction are the most important risk factors for CHD. In the early stages of CHD, certain risk factors such as hypercholesterolaemia, hypertension, chemical stimuli, and obesity can promote endothelial cell damage. After that, macrophages are aggregated to the injury sites, subsequently taken up by oxidized low density lipoprotein, transformed to foam cells, ultimately produce the early atherosclerotic plaques (Wang et al. 2015), and causing the impaired coronary microvascular function. In addition, ventricular hypertrophy, thrombus, myocardial oedema and smooth muscle dysfunction may also cause impairment of coronary microvascular function (Gutiérrez et al. 2013).

Inflammation plays an important role in CHD. Pro-inflammatory cytokines and activated neutrophils, can cause instability of atherosclerotic plaque, thrombosis, platelet aggregation (Lyu et al. 2015), and induce the damaged endothelial cells (Zhang and Li 2018). The transcription factor nuclear factor-kB (NF-κB) can increase the level of pro-inflammatory cytokines and exacerbate the inflammatory reaction. During the process of inflammation, macrophages secrete a large amount of matrix metalloprotein-9 (MMP-9), which plays a key role in the process of basal membrane neutrophil migration, vascular remodelling, platelet production, and plaque rupture (Busti et al. 2010). MMP-9 can degrade collagen and allow smooth muscle cells to migrate inside blood vessels, which can accumulate other cellular material, leading to tissue ischaemic events and atherosclerosis (Han et al. 2018). In addition, reactive oxygen species (ROS) are also an important factor in CHD. Excessive ROS can be accompanied by the production of reactive nitrogen species (RNS), a strong oxidant produced by the reaction of nitric monoxide (NO) and O2⁻ (Lubos et al. 2008). Under inflammatory conditions, the production of NO greatly increased, combined with other ROS, contributing to the formation of oxidative stress,
In the development of CHD, PI3K/AKT pathway shows an extremely important role. But it works two ways. On the one hand, it can serve to repress cardiomyocytes apoptosis by inhibiting the activities of pro-apoptotic proteins and protecting the heart against ischemia-reperfusion injury; on the other hand, it can actively promote platelet aggregation (Feng et al. 2019), involved in physiological cardiac hypertrophy (Song et al. 2015) and lead to the formation of unstable atherosclerotic plaques prone to rupture (Zhai et al. 2014). Phosphoinositide 3-kinase (PI3K) activates protein kinase B (AKT), promotes its phosphorylation, regulates the transcriptional activity of NF-kB, or affects the upstream pathways (Hoesel and Schmid 2013). After activation, PI3K/AKT induces the expression of MMP-9, and enhanced chemokines released by endothelial cells (Dilly et al. 2013). Beyond that, activated AKT can directly phosphorylate endothelial nitric oxide synthase (eNOS), thereby promoting NO production (Abeyrathna and Su 2015), and give rise to oxidative stress injury. Therefore, selective inhibition of PI3K is of great significance in prevention and cure CHD.

In recent years, more and more traditional Chinese herbal preparations have been used to treat cardiovascular diseases. As a Traditional Chinese Medicine formula, Naoxintong capsule (NXT) was approved by the Sino Food and Drug Administration (Z20025001), and is currently used as a hospital prescription. NXT was developed from Buyang Huanwu decoction and is composed of 16 ingredients (Table 1). It has been widely used to treat CHD for more than 20 years in China (Yang et al. 2017). NXT can exert a cardioprotective effect by inhibiting NLRP3 inflammasome activation in the ischemia/reperfusion injury model (Wang et al. 2017). NXT can activate PPARα pathway to protected cardiomyocytes, inhibit cardiac hypertrophy, and retard atherosclerosis (Xu et al. 2016; Yuan et al. 2017; Wang et al. 2018). NXT also can reduce the NO level and the expression of inducible nitric oxide synthase (iNOS) in the vessel wall to treat atheromatous (Zhong et al. 2013). In clinics, NXT can be used for carotid atherosclerosis and cerebral infarction (Liang et al. 2018), partly abrogate the impaired protective effect of high-density lipoprotein (HDL) on endothelial function (Lv et al. 2016), and improve adverse cardiovascular events, atherosclerosis, and the expression of inflammatory factors in patients with acute coronary syndrome undergoing PCI, when combined with Danhong injection (Lv et al. 2015; Zhao et al. 2018).

Although NXT has been proven to be widely useful in the clinical treatment of cardiovascular diseases, due to the complex chemical composition, the mechanism of action and pharmacodynamic substance foundation of NXT are still unknown. In this study, based on previous work, we developed a multiple-high throughput screening method, explored the pharmacodynamic basis of NXT for inhibiting NF-kB, MMP-9, and NO, established effective active ingredients by in vitro screening, explored the effect of the active components on PI3K/AKT signalling pathway, identified the possible mechanism of NXT in prevention and cure CHD, and provide a theoretical basis for the clinical application of NXT.

Materials and methods

Sample preparation

NXT (1 g) (Heze Buchang Pharmaceutical Co., Ltd., Heze, China) was ultrasonically dissolved in 10 mL of 75% methanol (Merck, Darmstadt, Germany) for 10 min, centrifuged to obtain the supernatant and stored at −20°C. In a previous study, we identified 81 major compositions in NXT by Ultra-performance liquid chromatography/quadrupole time-of-flight (UPLC/Q-TOF) (Ma et al. 2016b). Based on the pre-existing condition, UPLC fractions were collected every 30 s and vacuum dried. The residues were store at −20°C for the follow-up experiment.

Cell culture

Human embryonic kidney cells (HEK 293 cells, American Type Culture Collection, Rockville, MD, USA) were cultured in high-glucose Dulbecco’s modified Eagle’s Medium (DMEM) ( Biological Industries Israel Beit Haemek Ltd., Israel), including 10% foetal bovine serum (FBS) ( Biological Industries Israel Beit Haemek Ltd., Israel), 0.1 mg/mL streptomycin and 100 U/mL penicillin ( Biological Industries Israel Beit Haemek Ltd., Israel). Cells passaged when they reached 70–80% confluence.

Human umbilical vein endothelial cell line EA.hy926, obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), cultured similarly to HEK 293 cells. All the cells were confluent in 96-well plates for 12 h before use.

Cell viability assay

Cell viability was measured by the MTT (Sigma Aldrich, Steinheim, Germany) assay. After incubation with drugs, cells were treated with MTT (20 μL, 0.5 mg/mL) for 4 h. Formazan crystals were dissolved in 150 μL DMSO by shaking for 15 min, and measured the viability at 490 nm.

Screening of anti-NR-kB components

Transfection

HEK 293 cells were seeded into a 96-well plate and transfected when the cell confluence was 50–70%. Cells were transfected with the NF-kB luciferase reporter plasmid PGL 4.32 and the Renilla luciferase reporter vector plasmid pRL-TK at 100 and 9.6 ng per well, respectively. Transfection was performed for 24 h by using the transfection reagent PEI (PEI: pGL4.32 = 8:1, W/W) before drug treatment.
Figure 1. Cell viability assay. (A) TNF-α treated 293 T cell viability detected by MTT assay. (B) LPS treated 293 T cell viability detected by MTT assay. (C) H2O2 treated EA hy926 cell viability detected by MTT assay. The results are the mean±SEM (n = 6 per group); **p < 0.01, ***p < 0.001, versus the control group; *p < 0.05, *p < 0.01, versus the model group.

Dual-luciferase assay

Hek 293 cells were incubated with DMEM containing TNF-α (20 ng/mL, Sigma, St. Louis, MO, USA) for 6 h. The cells pre-treated with dexamethasone (DEX, 10 μmol/L, Sigma, St. Louis, MO, USA) and drugs (diluted by DMEM, the concentration was identical with the effective concentration of NXT we had detected before) for 24 h after transfection. All the chemical standards were purchased from Chengdu Must Bio-technology Co., Ltd. (Chengdu, China). Dual-Luciferase Reporter Assay Kit was used to detect the transcriptional activity of NF-κB.

Screening of anti-MMP-9 components

EA.hy926 cells were treated for 24 h to DEX (10 μmol/L) plus lipopolysaccharides (LPS, 10 μg/mL, Peprotech, Rocky Hill, CT, USA), drugs (diluted by DMEM, the concentration was identical with the effective concentration of NXT) plus LPS (10 μg/mL), LPS (10 μg/mL), or DMEM before detection. Human MMP-9 ELISA kits (R&D Systems, Minneapolis, MN, USA) were used to measure the expression of MMP-9. The absorbance was measured at 450 nm.

Screening of anti-NO components

After incubation in 96-well plates, EA.hy926 cells were exposed for 24 h to Nimodipine (NIM, 10 μmol/L, Sigma, St. Louis, MO, USA) plus H2O2 (100 μmol/L, Sigma, St. Louis, MO, USA), drugs (diluted by DMEM) plus H2O2 (100 μmol/L), H2O2 (100 μmol/L), or cell culture medium before detection. Treatments were performed at room temperature in the absence of light. Human NO ELISA kits (R&D Systems, Minneapolis, MN, USA) were used to measure the release of NO from the supernatants of EA.hy926 cells after stimulation. The absorbance was measured at 450 nm.

Western-blot

Cells collected after incubation with drugs were prepared with RIPA buffer (Solarbio, Beijing, China). Proteins were separated and transferred to PEGF membranes (GE Healthcare Life Science, Boston, MA, USA), and incubated with different primary antibodies overnight at 4 °C. The HRP-conjugated secondary antibody was incubated for 50 min, and followed by detection with chemiluminescence (ECL, ProteinSimple, Santa Clara, CA, USA). All the antibodies were bought from Cell Signalling Technology (Danvers, MA, USA).

Target prediction of active ingredients from NXT

We obtained the 3D structures of potential protein targets from Protein Data Bank (http://www.rcsb.org/pdb). The SYBYL software was used to construct and minimize the molecules and potential targets, and then docked by AutoDock version 4.2 using a hybrid Lamarckian genetic algorithm (LGA). Using the Molecular Operating Environment (MOE 2015.10) software was used to dock the molecules into the active site of the potential protein.

Statistical analysis

All the results were expressed as the mean values±standard error of the mean (SEM). Data were evaluated by one-way analysis of variance (ANOVA) with the Tukey’s test for comparisons between groups using SPSS 16.0. Probability values of less than 0.05 were considered to be significant.

Result

Cell viability

According to Figure 1(A), the viability of the model group (treated with TNF-α) was decreased (**p < 0.01 vs. control). When it was treated by NXT and DEX, the cell viability increased significantly (*p < 0.05 for 10 μg/mL, 100 μg/mL NXT vs. model, ***p < 0.01 for 1 μg/mL NXT and DEX vs. model). In Figure 1(B), the viability of the LPS (10 μg/mL) treated group was decreased (**p < 0.001 vs. control) but the intervention group could improve the viability of cells (**p < 0.01 for DEX and 10 μg/mL NXT vs. model). H2O2 (100 μmol/L) could reduce the viability (**p < 0.01 vs. control); the cell viability increased when treated by NIM and NXT (*p < 0.05 for NIM and 1 μg/mL, 10 μg/mL NXT vs. model) (Figure 1(C)).

Screening of NF-κB inhibitory active ingredients in NXT

In Figure 2(A), we screening the optimal concentration of NXT to inhibit NF-κB by dual-luciferase assay. NXT (1, 10 μg/mL) could decrease the over-expression of NF-κB induced by TNF-α (***p < 0.001, **p < 0.01, with IC50 values of 0.1178 μg/mL),
and 1 μg/mL was more effective. Through the dual-luciferase assay (Figure 2(B)), the UPLC fraction in 12 time periods we collected could inhibit NF-κB: 2–2.5 min, 3–3.5 min, 3.5–4 min, 4–4.5 min, 5.5–6 min, 6–6.5 min, 7.5–8 min, 10–10.5 min, 10.5–11 min, 12–12.5 min, 12.5–13 min and 13.5–14 min. The effective fractions were analysed by mass spectrometry, and we discovered 26 ingredients as potential NF-κB inhibitors: phenylalanine, danshensu, senkyunolide B, senkyunolide C, protocatechuic aldehyde, mulberroside A, gallicin, hydroxysafflor yellow A, 7-hydroxycoumarin, quercetin-7-O-glucoside, rutin, calycosin, calycosin-7-O-glucoside, ferulic acid, salvianolic acid B, ononin, senkyunolide F, salvianolic acid E, pratensein, hydroxyl calendic acid, trans-oxyresveratrol, and salvianolic acid F. The anti-NF-κB ingredients in NXT were verified (Figure 2(C)), and 21 ingredients were identified that can inhibit NF-κB (Table 2, Figure 2(D)).

The screening of MMP-9 inhibitory active ingredients in NXT
In Figure 3(A), we screening the effective concentration of NXT to inhibit MMP-9. NXT (1, 10 μg/mL) can inhibit MMP-9 induced by LPS (**p < 0.01, *p < 0.05, with an IC50 value of 0.1182 μg/mL), and 10 μg/mL was the optimum concentration. Through the MMP-9 inhibition screening (Figure 3(B)), the UPLC fraction in 8 time periods found activation at: 5–5.5, 5.5–6, 7.5–8, 9.5–10 min and 12.5–13 min, and 22 compounds were discovered as potential MMP-9 inhibitors: phenylalanine, danshensu, senkyunolide B, senkyunolide C, protocatechuic aldehyde, mulberroside A, vanillic acid, benzoic acid, epicatechin, quercetin-7-O-glucoside, rutin, calycosin, calycosin-7-O-glucoside, ferulic acid, salvianolic acid B, ononin, senkyunolide F, salvianolic acid E, pratensein, hydroxyl calendic acid, trans-oxyresveratrol, and salvianolic acid F. The anti-MMP-9 ingredients in NXT were verified (Figure 3(C)), and 16 ingredients were identified that can inhibit MMP-9 (Table 3, Figure 3(D)).

The screening of NO inhibitory active ingredients in NXT
In Figure 4(A), NXT (1, 10 μg/mL) inhibits NO (**p < 0.01, *p < 0.05, with an IC50 value of 0.1094 μg/mL), and 10 μg/mL was the most effective concentration as an antioxidant. Through the NO inhibition screening (Figure 4(B)), the UPLC fraction in 7 time periods found activation at: 5–5.5, 5.5–6, 7.5–8, 10–10.5, 12.5–13, 13.5–14, and 18–18.5 min. Seventeen compounds were discovered as potential NO inhibitors: catechin, albiflorin, quercetin-7-O-glucoside, rutin, calycosin, calycosin-7-O-glucoside, ferulic acid, salvianolic acid B, ononin, senkyunolide F, salvianolic acid E, formononetin, astragaloside IV, salvianolic acid F, trijuganone B, tanshinone IIA, and angelicide; the biological active ingredients in NXT were verified (Figure 4(C)). Thirteen ingredients were identified that cause the NO inhibition (Table 4, Figure 4(D)).

Molecular docking of the biological activity ingredients in NXT with PI3K
As a result of screening, six components in NXT were determined to inhibit NF-κB, MMP-9, and NO: salvianolic acid B,
ferulic acid, calycosin, salvianolic acid E, ononin, and salvianolic acid F (Figure 5(A)). The inhibition rate of the biological active ingredients is shown in Table 5. Based on reports, PI3K/AKT pathway plays a crucial role in CHD, so we determined whether the biological activity of NXT was related to the PI3K/AKT signalling pathway. We docked six biological active ingredients to the binding site of PI3K (PDB: 5ITD), and found that all the ingredients had better associativity with PI3K. The binding energy is shown in Table 6. After that, we analysed and displayed a 3D map (Figure 5(B)) indicating the interaction of the biological active ingredients in NXT with PI3K. All the reactions take place in PIK helical domain and PI3K/PI4K domain, which are the activity region of PI3K. The ingredients established hydrogen bonds with the active site amino acids, such as ASP-

Table 2. NF-κB-inhibiting compounds identified in Naoxintong Capsules.

| Peak No. | RT (min) | Identification | Mode | MS (m/z) | Composition | Herbal source |
|---------|----------|----------------|------|----------|-------------|---------------|
| 16      | 2.459    | Danshensu      | Neg  | 198.0501 | C_9H_10O_5  | RSM           |
| 18      | 3.438    | Senkyunolide B | Neg  | 204.2374 | C_12H_10O_3 | RCX           |
| 19      | 3.456    | Senkyunolide C | Neg  | 204.2374 | C_12H_10O_3 | RCX           |
| 20      | 3.600    | Protocatechuic aldehyde | Neg | 138.1185 | C_7H_6O_3   | RSM, RC       |
| 21      | 3.974    | Mulberrieside A| Neg  | 568.5277 | C_26H_32O_14| RM            |
| 22      | 4.122    | Gallicin       | Neg  | 184.1453 | C_9H_10O_5  | RSM           |
| 23      | 4.230    | Hydroxysafflor yellow A | Pos/Neg | 612.5364 | C_23H_16O_9 | FC            |
| 24      | 5.952    | Rutin          | Neg  | 610.5203 | C_23H_16O_9 | RA            |
| 25      | 5.907    | Calycosin      | Neg  | 284.2679 | C_12H_10O_3 | RA            |
| 26      | 5.989    | Ferulic acid   | Neg  | 194.1815 | C_12H_10O_3 | RA, RCX, RAS, RAB |
| 27      | 6.321    | Paeoniflorin   | Pos  | 480.466  | C_12H_10O_3 | RPR           |
| 28      | 7.540    | Salvianolic acid B | Neg/Pos | 718.6220 | C_23H_16O_9 | RSM           |
| 29      | 7.688    | Ononin         | Pos  | 430.4107 | C_12H_10O_3 | RA, CS        |
| 30      | 7.763    | Senkyunolide F | Pos  | 206.1017 | C_12H_10O_3 | RCX, RAS      |
| 31      | 7.855    | Salvianolic acid E | Neg | 718.1512 | C_23H_16O_9 | RSM           |
| 32      | 10.240   | Formononetin   | Pos/Neg | 268.2580 | C_12H_10O_3 | RA            |
| 33      | 10.405   | Astragaloside IV | Neg | 784.4633 | C_26H_32O_14| RA            |
| 34      | 10.590   | Senkyunolide H | Neg  | 220.2305 | C_12H_10O_3 | RCX           |
| 35      | 10.978   | Astragaloside II | Neg | 826.4701 | C_23H_16O_9 | RA            |
| 36      | 12.198   | Senkyunolide A | Pos  | 192.2516 | C_12H_10O_3 | RCX           |
| 37      | 12.975   | Salvianolic acid F | Neg | 314.0735 | C_12H_10O_3 | RSM           |
| 38      | 13.917   | Trijuganone B  | Pos  | 280.1107 | C_12H_10O_3 | RSM           |

Figure 3. Bioactivity analysis of NXT on anti-MMP-9. (A) Effect of NXT on MMP-9 activation in LPS-stimulated 293 T cells. (B) The screening of potential MMP-9 inhibitory active ingredients in NXT. (C) Verification the MMP-9 inhibition active ingredients in NXT. (D) Chemical structures of the MMP-9 inhibitory active ingredients in NXT. The results are the mean ± SEM (n = 6 per group); ***p < 0.001 versus the control group; *p < 0.05, **p < 0.01, ***p < 0.001, versus the model group.
Table 3. MMP-9-inhibiting compounds identified in Naoxintong Capsules.

| Peak No. | RT (min) | Identification   | Mode  | MS (m/z) | Composition   | Herbal source |
|---------|----------|------------------|-------|----------|--------------|---------------|
| 16      | 2.459    | Danshensu        | Neg   | 198.0501 | C<sub>9</sub>H<sub>10</sub>O<sub>5</sub> | RSM           |
| 18      | 3.438    | Senkyunolide B   | Neg   | 204.2374 | C<sub>12</sub>H<sub>14</sub>O<sub>3</sub> | RCX           |
| 19      | 3.456    | Senkyunolide C   | Neg   | 204.2374 | C<sub>12</sub>H<sub>14</sub>O<sub>3</sub> | RCX           |
| 20      | 3.600    | Protocatechualdehyde | Neg | 138.1185 | C<sub>6</sub>H<sub>6</sub>O<sub>3</sub> | RSM, RC       |
| 21      | 3.970    | Mulberroside A   | Neg   | 568.5277 | C<sub>25</sub>H<sub>30</sub>O<sub>14</sub> | RM            |
| 22      | 4.565    | Vanillic acid    | Neg   | 168.1459 | C<sub>9</sub>H<sub>8</sub>O<sub>4</sub> | RCX, RPR      |
| 26      | 4.694    | Benzoic acid     | Neg   | 122.1209 | C<sub>7</sub>H<sub>6</sub>O<sub>2</sub> | RPR           |
| 28      | 5.970    | Calycosin        | Neg   | 284.2679 | C<sub>14</sub>H<sub>22</sub>O<sub>7</sub> | RA            |
| 34      | 5.989    | Ferulic acid     | Neg   | 194.1815 | C<sub>14</sub>H<sub>22</sub>O<sub>7</sub> | RA, RCX, RAS, RAB |
| 43      | 7.540    | Salvianolic acid B | Neg/Pos | 718.6220 | C<sub>36</sub>H<sub>30</sub>O<sub>16</sub> | RSM           |
| 44      | 7.688    | Ononin           | Pos   | 430.4107 | | RA, CS        |
| 46      | 7.763    | Senkyunolide F   | Pos   | 206.1017 | C<sub>12</sub>H<sub>14</sub>O<sub>3</sub> | | RCR, RAS, RAB |
| 46      | 7.855    | Salvianolic acid E | Neg  | 718.1512 | C<sub>36</sub>H<sub>30</sub>O<sub>16</sub> | RSM           |
| 52      | 9.611    | Hydroxyl calendic acid | Neg | 294.4342 | C<sub>14</sub>H<sub>22</sub>O<sub>7</sub> | SP            |
| 53      | 9.648    | Trans-Oxyresveratrol | Pos  | 244.2435 | C<sub>14</sub>H<sub>22</sub>O<sub>7</sub> | RM            |
| 66      | 12.975   | Salvianolic acid F | Neg  | 314.0735 | C<sub>17</sub>H<sub>16</sub>O<sub>6</sub> | RSM           |

Figure 4. Bioactivity analysis of NXT on anti-NO. (A) Effect of NXT on NO activation in H<sub>2</sub>O<sub>2</sub>-stimulated EA hy926 cells. (B) The screening of potential NO inhibitory active ingredients in NXT. (C) Verification the NO inhibition active ingredients in NXT. (D) Chemical structures of the NO inhibitory active ingredients in NXT. The results are the mean ± SEM (n = 6 per group); *p < 0.05, **p < 0.01, ***p < 0.001, versus the model group.

Table 4. NO-inhibiting compounds identified in Naoxintong capsules.

| Peak No. | RT (min) | Identification   | Mode  | MS (m/z) | Composition   | Herbal source |
|---------|----------|------------------|-------|----------|--------------|---------------|
| 28      | 5.157    | Catechin         | Neg   | 290.2674 | C<sub>11</sub>H<sub>14</sub>O<sub>5</sub> | RPR           |
| 29      | 5.212    | Albidflorin      | Neg   | 480.4653 | C<sub>32</sub>H<sub>32</sub>O<sub>11</sub> | RPR           |
| 31      | 5.952    | Rutin            | Neg   | 610.5203 | C<sub>28</sub>H<sub>30</sub>O<sub>16</sub> | RA            |
| 32      | 5.970    | Calycosin        | Neg   | 284.2679 | C<sub>16</sub>H<sub>20</sub>O<sub>5</sub> | RA            |
| 34      | 5.989    | Ferulic acid     | Neg   | 194.1815 | C<sub>14</sub>H<sub>22</sub>O<sub>7</sub> | RA, RCX, RAS, RAB |
| 43      | 7.540    | Salvianolic acid B | Neg/Pos | 718.6220 | C<sub>36</sub>H<sub>30</sub>O<sub>16</sub> | RSM           |
| 44      | 7.688    | Ononin           | Pos   | 430.4107 | | RA, CS        |
| 46      | 7.855    | Salvianolic acid E | Neg  | 718.1512 | C<sub>36</sub>H<sub>30</sub>O<sub>16</sub> | RSM           |
| 54      | 10.240   | Formononetin     | Pos/ Neg | 268.2580 | C<sub>14</sub>H<sub>22</sub>O<sub>7</sub> | RA            |
| 55      | 10.405   | Astragaloside IV | Pos   | 784.4633 | C<sub>19</sub>H<sub>30</sub>O<sub>13</sub> | RA            |
| 66      | 12.975   | Salvianolic acid F | Neg  | 314.0735 | C<sub>17</sub>H<sub>16</sub>O<sub>6</sub> | RSM           |
| 71      | 13.917   | Trijuganone B    | Pos   | 280.1107 | C<sub>14</sub>H<sub>22</sub>O<sub>7</sub> | RSM           |
| 76      | 18.076   | Tanshinone IIA   | Pos   | 294.3430 | C<sub>16</sub>H<sub>20</sub>O<sub>5</sub> | RSM           |
843, SER-840, ARG-808 and HIS-670. It is worth mentioning that all the ingredients could interact with ARG-808; it may be the major active site of PI3K.

Inhibition of the PI3K/AKT pathway by the biological activity ingredients in NXT

We explored the role of the biological active ingredients in NXT with the PI3K/AKT pathway under different conditions. In Figure 6(A), 293T cells were treated by TNF-α; all the ingredients could inhibit PI3K/AKT pathway, especially ferulic acid, salvianolic acid E and salvianolic acid F. Salvianolic acid B and salvianolic acid E were found more effective than the other ingredients in the LPS treated model to inhibited PI3K and p-AKT (Figure 6(B)). In Figure 6(C), oxidative stress model was induce by H2O2 and intervened using the biological active ingredients. The level of PI3K and p-AKT significantly decreased in the intervention group in comparison to the model, and calycosin, salvianolic acid B, and salvianolic acid E were much more effective than other ingredients.

Discussion

The treatment of cardiovascular disease by NXT may be related to its antioxidative and anti-inflammatory effects. According to Li et al. (2012), long-term administration of NXT can inhibit the expression of NF-κB, decrease the content of MMP-9, in MMP-9/TIMP-1 serum may inhibit inflammation, and the incidence of critical lesions in patients with CHD could be reduced. In addition, NXT also has a protective effect on myocardial peroxidation damage caused by H2O2 (Zhang et al. 2013). It can pre-regulate enhanced antioxidant functions by increasing total superoxide dismutase, total antioxidative capacity, and catalase activity, reducing the production of malondialdehyde and reactive oxygen species. However, due to the large number of compositions and the complication of the interaction between various components, the basis and mechanism of the pharmacodynamics are unclear. Defining the basis of pharmacodynamics is not only a prerequisite for elucidating the mechanism of traditional Chinese medicines, but also an important basis for quality control, and provides a new material basis for the discovery of active compounds (Zhang et al. 2008).

Standard methods can screen effective ingredients by extracting, separating, purifying, and then combining in vivo
experiments. This requires a large amount of manpower and material resources, as well as a long experiment period, and has a poor specificity. In recent years, systematic biological screening systems based on DNA, proteins, membrane receptors and cells have become an effective method for screening (Warzecha et al. 2012). UPLC/Q-TOF, combined with cell biological activity detection, has been directly applied to the screening of effective active ingredients in natural medicines (Zhou et al. 2017). Compared to the traditional screening technology, it has the characteristics of fast, trace and high specificity, and has gradually replaced the traditional screening technology.

In this study, based on our previous work, we established a multi-screening method using UPLC/Q-TOF combined with high-throughput screening, and linking chemomics with systems biology to build an integrated systems biology. We have screened 21 components that could be NF-\(\kappa\)B inhibitors, 16 active ingredients can inhibit the expression of MMP-9 and 13 ingredients have antioxidative effects in NXT, comprehensively revealing the correlation between the chemical constituents of NXT and the response of the body’s disease system.

Interestingly enough, the drug’s activity in inhibiting NF-\(\kappa\)B, MMP-9, and NO was not dose-dependent for all doses tested. When the concentration of NXT reaches a certain level, the protective effect on cells will gradually decline. The reasons for this situation may be concerned with the following aspects: first of all, NXT has different protective mechanisms for cell damage. It imposes a significant inhibitory effect on NF-\(\kappa\)B, and thus causes a relatively low effective concentration. In addition, dose–effect relationship is found between the efficacy and the dose within a certain range, but it becomes insignificant when the concentration exceeds the certain range. In addition, excessive drug concentration will give rise to cytotoxicity, causing the cell state to be affected and reducing the protective effect of drugs on cells. In subsequent studies, the optimal dose will be screened to ensure drug’s cytoprotective effect at the optimal concentration.

According to our results, we found six components in NXT that inhibit NF-\(\kappa\)B, MMP-9, and NO (salvianolic acid B, ferulic acid, calycosin, salvianolic acid E, ononin, and salvianolic acid F), which are the main active ingredients in NXT. As a matter of record, ferulic acid has antioxidation and anti-inflammatory effects, and is widely used in the treatment of cardiovascular diseases (Mancuso and Santangelo 2014; Doss et al. 2016). Calycosin and ononin can reduce TSLP production by regulating NF-\(\kappa\)B activation to attenuated allergic inflammation (Shen et al. 2014). In addition, they can also inhibit the expression of MMP-9 while purifying NO, in order to protect the blood–brain barrier integrity of ischaemic brain tissue (Fu et al. 2014; Quan et al. 2015). Salvianolic acid B could inhibit NF-\(\kappa\)B and AP-1 DNA binding activities activated by TNF-\(\kappa\) (Zhou et al. 2005). It also can block the activation of NF-\(\kappa\)B, JNK, and ERK1/2 signalling pathways to inhibit MMP-9 (Zhou et al. 2005; Ma et al. 2015), and has an antioxidative effect (Jiang YF et al. 2015). In past

Figure 6. The active ingredients in NXT could inhibit the PI3K/AKT pathway. (A) Western-blotting for the cells treated by TNF-\(\kappa\). (B) Western-blotting for the cells treated by LPS. (C) Western-blotting for the cells treated by H\(_2\)O\(_2\). The results are the mean ± SEM (n = 3 per group); **\(p < 0.01\), ***\(p < 0.001\) versus the control group; *\(p < 0.05\), **\(p < 0.01\), ***\(p < 0.001\), versus the model group. Lane 1: control, lane 2: model, lane 3: positive drugs (DEX for A, B and NIM for C), lane 4: NXT, lane 5: Calycosin, lane 6: Ononin, lane 7: Ferulic acid, lane 8: Salvianolic acid B, lane 9: Salvianolic acid E and lane 10: Salvianolic acid F.
studies on salvianolic acid components, the focus of attention has been on the higher content of salvianolic acid A and B (Jiang X et al. 2015), but the pharmacological activities of salvianolic acids E and F, which have analogous structures with the basal body of salvianolic acid B, are rarely reported. This study is the first report on the inhibition of NF-kB, NO, and MMP-9 by salvianolic acid E and salvianolic acid F simultaneously. Both of them had significant anti-inflammatory and antioxidative activity, however salvianolic acid F was much better than salvianolic acid E. These ingredients are the main effective active substances in NXT, which is the main reason for treatment of cardiovascular diseases. In addition, the co-action targets of the six components are also explored in this paper. PI3K/AKT signalling pathway plays an important role in various diseases such as inflammation, cancer, and cardiovascular diseases. The active ingredients in NXT can be targeted to PI3K, reduce the over expression from inflammation and oxidative stress, thereby inhibiting the expression of p-AKT, in favour of anti-platelet aggregation, inhibit the production of unstable atherosclerotic plaques, and protect the coronary microvascular function. That may be the mechanism of NXT in the prevention and cure of CHD.

In this study, we finally established the pharmacodynamic substance basis of NXT by multiple screening, clarified the healing effect and molecular mechanism of its multi-component synergy, and explored the synergistic relationship between its active ingredients. This article provides a theoretical basis for the treatment of coronary heart disease and clinical application of NXT, which is conducive to the development of new drugs and has a great significance in promoting the modernization and internationalization of traditional Chinese medicine.

Conclusions

NXT proves effective by inhibiting NF-kB, MMP-9, and NO which is a key in the treatment of CHD. We found six components in NXT determined to inhibit NF-kB, MMP-9, and NO: ferulic acid, calycosin, ononin, salvianolic acid B, salvianolic acid E and salvianolic acid F, which come from the traditional Chinese medicine RA, RCX, RAS, RAB, CS, and RSM. In addition, all of the biological active ingredients in NXT could inhibit the PI3K/AKT pathway, this should be the treatment mechanism of CHD.

Disclosure statement

The authors declare that they have no competing interests.

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