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ORIGINAL RESEARCH

Naoxintong Is Involved in the Coagulation Regulation of Warfarin Through the MAPK Pathway

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Objective: To explore the effect of Naoxintong (NXT) on warfarin anticoagulation therapy and its potential mechanism.

Methods: TCSMP, SwissTargetprediction, SuperPred, SEA, and Batmanic-TCM were used to search for active ingredients and targets of NXT and warfarin; the DisGENT database was used to find disease targets of coagulation disorders. Cytoscape software was applied to construct the "drug-target"network; the protein interaction network (PPI) was used to study the protein-protein interaction. GO and KEGG were used for functional analysis. The effect of NXT on warfarin anticoagulation was then tested in rats by analyzing coagulation factors in blood before and after drug administration. The expression of MAPK in the liver tissue was determined by Western blot.

Results: The top five components of NXT affecting warfarin anticoagulation degree value were MOL000098, MOL000422, MOL000006, MOL000358, and MOL000449. TP53, AKT1, SRC, TNF, HSP90AA1, STAT3, JUN, IL6, EGFR, and ESR1 were the core targets of NXT, while MAPK9, MAP3K5, MAPK8, and MAPK1 in the MAPK family were important targets of NXT in the coagulation process. In vivo testing indicated that NXT may be able to participate in the regulation of the warfarin coagulation process through multiple targets and multiple pathways, which may be related to MAPK.

Conclusion: Our data suggests that NXT is involved in the coagulation regulation of warfarin through the MAPK pathway.

Keywords: Naoxintong, Warfarin, coagulation dysfunction, network pharmacology, mechanism research

Introduction

Coagulopathy, often broadly defined as any derangement of hemostasis resulting in either excessive bleeding or clotting, is a blood clotting disorder that can be congenital or acquired (caused by defective synthesis of blood clotting factors and blood platelets or by the absorption of antibodies that attack blood clotting functions). Early recognition of coagulopathy is necessary for its prompt correction and successful management.[1] Coagulopathy has a genetic basis; a recent study identified FAB2, a protein related to the transport of long-chain fatty acids, as contributing to the risk of pulmonary embolism in Chinese individuals.[2]

Warfarin is an oral anticoagulant commonly used to treat and prevent blood clots. Warfarin inhibits the vitamin K epoxide reductase complex 1, an essential enzyme for activating the vitamin K available in the body, thereby reducing the synthesis of active clotting factors.[3] Warfarin is an important drug for anticoagulant therapy, and it is the only oral drug choice for anticoagulant therapy in some severe valve stenosis and after metal valve replacement. Warfarin is not only widely used for anticoagulation of atrial fibrillation and stroke prevention but also has important application value in pulmonary embolism and various surgical treatments.[4–6] Warfarin is also listed in the World Health Organization's List of Essential Medicines.[7] However, a high risk of bleeding and severe kidney damage are some of its major side effects.[8,9] Even though novel anticoagulant treatments have been developed over the years, such as point-of-care testing[10] and administration of coagulation factor concentrates[11] aimed to tailor the hemostatic therapy to each patient and thus reduce the risks of over- or under-transfusion, warfarin remains the only choice for patients with severe valve disease and after metal valve replacement.

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Traditional Chinese medicine (TCM), which incorporates herbal medicines and mind-body practices, has been applied for centuries to treat diseases. NXT capsule is a TCM approved by the China Food and Drug Administration (CFDA) for preventing and treating cerebrovascular and cardiovascular diseases.[12–14] Clinical studies have shown that NXT is effective in patients with cerebral infarction,[12,13] transient ischemic attack,[15] carotid atherosclerosis,[13] and vertebra-basilar insufficiency. Also, NTX seems to affect the coagulation process. For example, Lu et al[16] suggested that its application could reduce warfarin dosage because the international standard ratio (INR) was up to the standard. Feng et al[17] also pointed out that using NXT and warfarin simultaneously has different effects on prothrombin time (PT) and other parameters. Nevertheless, only a few studies have investigated the effect of NXT combined with warfarin on coagulation. In addition, the specific mechanism of action and possible pathways involved in NTX are still not fully understood.

Dorgalaleh et al[18] reported that combining NXT and warfarin affects prothrombin time, INR, and other indicators. In this study, we explored the relationship network of "drug-active factor-target-signaling pathway-disease" of NXT participating in the anticoagulation process of warfarin by network pharmacology, as well as the possible mechanism of NTX effect on warfarin coagulation process. Network pharmacology is a multi-component, multi-targeted, and integrative methodology to identify the possible underlying causes of disease and the possible mechanisms of action of a drug or TCM preparation.[19–21]

Materials and Methods

Collection of Chemical Components and Target Prediction of NTX and Warfarin

All chemical constituents of NXT (peach kernel, mulberry branch, frankincense, myrrh, Niuxi, Jixueteng, *Astragalus*, safflower, cassia branch, Danggui, *Salvia miltiorrhiza*, Chuanxiong, red peony, leech, *Dipterocarpus*, and buthus) were retrieved from the TCM Systems Pharmacology (TCMSP) database (https://tcmspw.com/tcmsp.php), a professional and unique systems pharmacology tool designed for TCM.[22] Oral bioavailability (OB) ≥ 30% and drug-likeness (DL) ≥ 0.18 were the screening criteria. The target genes corresponding to active compounds of NXT were screened out from the TCMSP database. Obtained target genes were imported into UniProt (https://www.uniprot.org/) to search for their information, including the gene symbol and gene ID.

In order to identify targets of the active ingredients, the screened active compounds were subjected to various web servers and databases, including TCMSP, Swiss Target Prediction (http://www.swisstargetprediction.ch/), SuperPred (https://prediction.charite.de/index.php), and SEA (http://www.sea.ch/).

Collection of Blood Coagulation Dysfunction-Related Targets

GeneCards (https://www.genecards.org/) and OMIM (https://www.omim.org/) databases were searched to identify blood coagulation dysfunction-related targets; the keyword "blood coagulation dysfunction" was applied. The two databases' targets were integrated, and duplicated genes were eliminated. The disease target gene information was obtained by correcting it in the UniProt database (https://www.uniprot.org/).

Drug-Disease Target Prediction and Target Protein Interaction Network Construction (PPI)

The obtained drug component targets and disease targets were mapped to each other in a Venn diagram to obtain intersection genes. Then the Cytoscape software was used to construct the "drug-target" network. In order to further study the protein-protein interaction between NXT and warfarin in the treatment of coagulation disorders, the drug-intersection genes were uploaded to the interaction database String (https://string-db.org/) for protein interaction network (PPI) construction. The species was set to "Homo sapiens"; the minimum interaction score was set to 0.9 to ensure the credibility of this study; the other parameters were kept in the default Settings.

Bioinformatics (GO) Enrichment Analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) Signaling Pathway Analysis

Pathway and gene ontology enrichment were analyzed via the DAVID database. The gene lists were uploaded with OFFICIAL_GENE_SYMBOL and species Homo sapiens being selected at DAVID website https://david.ncifcrf.gov/summary.jsp, respectively. The target proteins studied in DAVID6.8GO gene functions of NXT and warfarin in the treatment of

coagulation disorders were annotated from three aspects: biological process (BP), cellular component (CC), and molecular Function (MF).

Animals and Grouping

Male SD rats, 6 weeks old, weighing 200 g, were obtained from Beijing Huafukang Biotechnology Company [SCXK (Beijing) 2019–0008, the experimental unit use license number is SYXK (Jiangxi)-2020-0001]. All the animals were housed in an environment with a temperature of 22 ± 1 °C, a relative humidity of 50 ± 1%, and a light/dark cycle of 12/12 hr. All animal studies (including the mice euthanasia procedure) were done in compliance with the regulations and guidelines of Nanchang University institutional animal care and conducted according to the AAALAC and the IACUC guidelines (Ethical review number: JJSDYRMYY-YXLL-2023-130).

After one week of adaptive feeding, animals were randomly divided into groups (6 rats per group): the control group, the NTX group, the warfarin group, and the combined group (NXT+warfarin). The control group was given a routine diet; the NTX group was given 1 g/kg/d NXT; the warfarin group was given a dose of 0.3 mg/kg/d, and the combined group was given NXT 1 g/kg/d + warfarin 0.3mg/kg/d; all substances were administrated by oral gavage.

Blood samples were collected from the tail vein into EDTA-containing tubes (Hebei Kangweishi Medical Technology Co., Ltd., Product Code: 22618858) 7 days after drug administration, and four coagulation parameters were detected. The coagulation factors (II, VII, IX, and X) levels were detected by ELISA before and 7 days after drug administration. Based on the network pharmacology research basis, the expression of MAPK in the liver tissue was determined.

Coagulation Tests

Four coagulation tests were performed using an animal automatic biochemical analyzer (SMT-120VP, Chengdu Smart Technology Co., LTD., China). Fibrinogen (Fib), thrombin time (TT), prothrombin time (PT), and activated partial thromboplastin time (APTT) were analyzed in each group.

Coagulation Factor ELISA Assay

The levels of rat coagulation factor II (F II), rat coagulation factor VII (F VII), rat coagulation factor IX (F IX), and rat coagulation factor X (F X) in the plasma of each group of rats before and after treatment were detected by ELISA kit following the manufacturer's instructions. The test kit information is as follows: Rat coagulation Factor II (FII) kit (JL12646, Jianglai Biology); Rat coagulation Factor VII (FVII) kit (JL21212, Jianglai Biology); Rat coagulation Factor IX (FIX) kit (JL31545, Jianglai Biology); Rat coagulation factor X (FX) kit (JL11817, Jianglai Biology). Briefly, venous blood was collected, and the plasma was separated from the blood by centrifugation at 5000 ×*g* for 10 minutes at room temperature. The ELISA kit was warmed to room temperature, and the standard solutions were prepared. The blank hole (no sample or enzyme substrate was added), standard hole, and sample hole were set up and incubated at 37°C for 1 hour. The plate was washed five times and treated with color developer for 15 minutes at 37°C, after which the absorbance value of each well was measured at 450 nm.

Western Blot

An equal amount of proteins was loaded and separated by 30% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were then incubated with the following primary antibodies: mouse anti-GAPDH (HC301, TransGen Biotech,1:2000), mouse anti p38MAPK (MA5-15116, Thermo, 1:1000), rabbit anti-P-P38/MAPK (Thr180/Tyr182, 28796-1-AP, Proteintech, 1:2000), and rabbit anti-ERK 1/2 (11257-1-AP, Proteintech, 1:1000) overnight at 4°C, and then with HRP conjugated goat anti-mouse IgG (H+L, GB23301, Servicebio, 1:2000) and HRP conjugated goat anti-rabbit IgG (H+L, GB23303, Servicebio, 1:2000) for 2h at room temperature.

Statistical Analysis

SPSS 22.0 was used for statistical analysis. All data obtained were analyzed by a *t*-test provided by GraphPad Prism 8.0.1 software. All P-values were two-sided, and $P<0.05$ was considered statistically significant.

Results

Active Ingredients and Drug-Component-Target Prediction of NXT and Warfarin

After applying OB ≥ 30% and DL ≥ 0.18 and excluding invalid components, 19 peach kernel components, three mulberry branch components, eight frankincense components, 45 myrrha components, 20 Niuxi components, 23 Jixueteng components, 17 *Astragalus membranaceus* components, 22 safflower components, six cinnamon twig components, two *Angelica sinensis* components, 60 *Salvia miltiorrhiza* components, six *chuangxiong* components, and 14 components of red peony root were identified. Next, we searched the Batmant-TCM database. After applying a score cutoff > 0.99 (LR=1626) and $P < 0.05$, 20 lumbricus components, 14 scorpion components, and 10 leech components were identified. Using probability > 0 as the inclusion criteria of the SwissTarget prediction, 96 targets were obtained, 65 and 40 targets were included by SuperPred and SEA, respectively, and 189 component targets were obtained after removing the weight.

A total of 4143 and 302 targets were obtained from GeneCards and OMIM databases, respectively. A total of 4397 targets related to coagulopathy-related genes were identified after merging. The obtained genes were corrected using the UniProt database. After selecting the intersection target genes of drug targets and coagulopathy, 479 intersection target genes of coagulopathy and NXT were identified, as well as 96 intersection target genes of coagulopathy and warfarin, which were the interaction target genes of coagulopathy caused by drugs.

In the Cytoscape mapping of NXT's drug-component-target data, the graph included 703 nodes and 2787 edges, and the top five core components with degree values were MOL000098, MOL000422, MOL000006, MOL000358, and MOL000449 (Figure 1). The network construction for warfarin included 97 nodes and 96 edges (Figure 2).

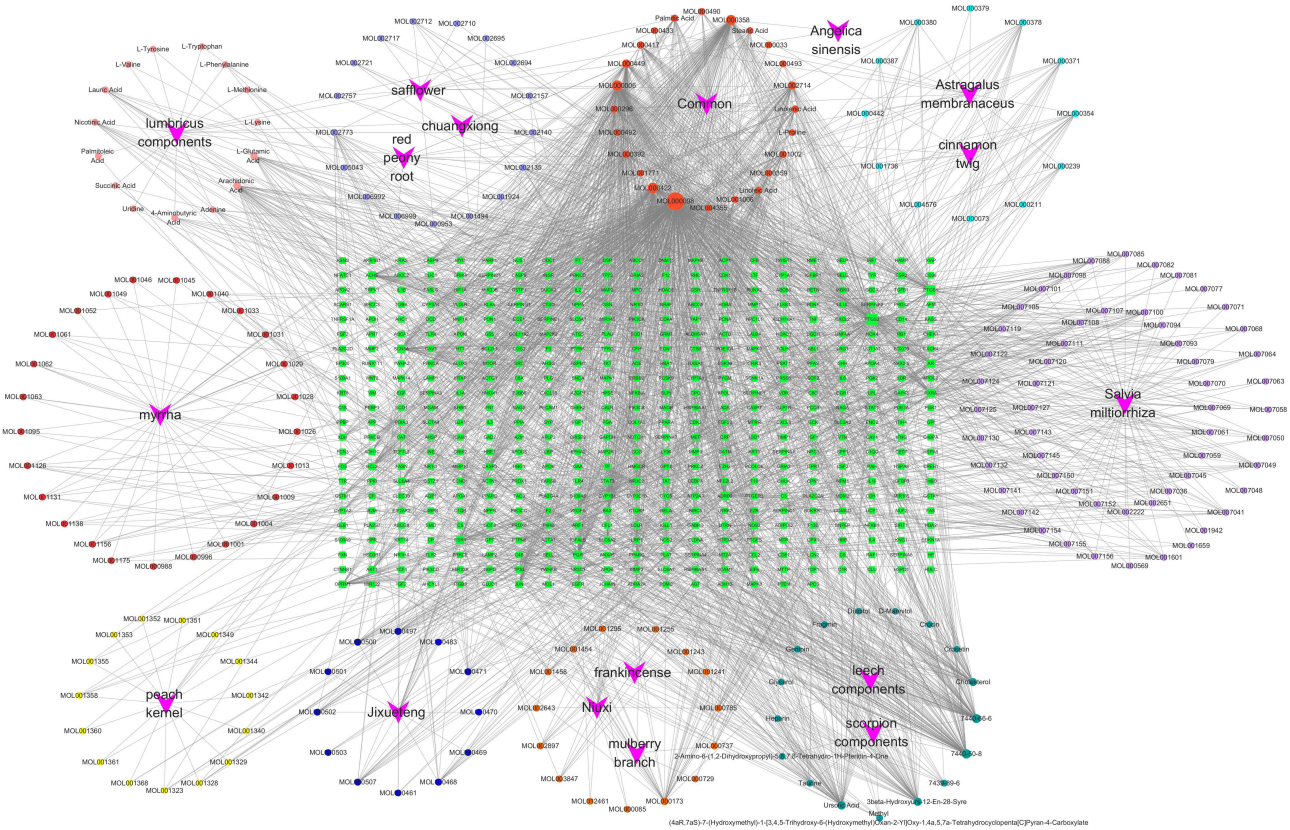

Figure 1 Drug-component-target action network of NXT. The size of the node shape represents the level of the degree value. The higher the degree value, the larger the node shape.

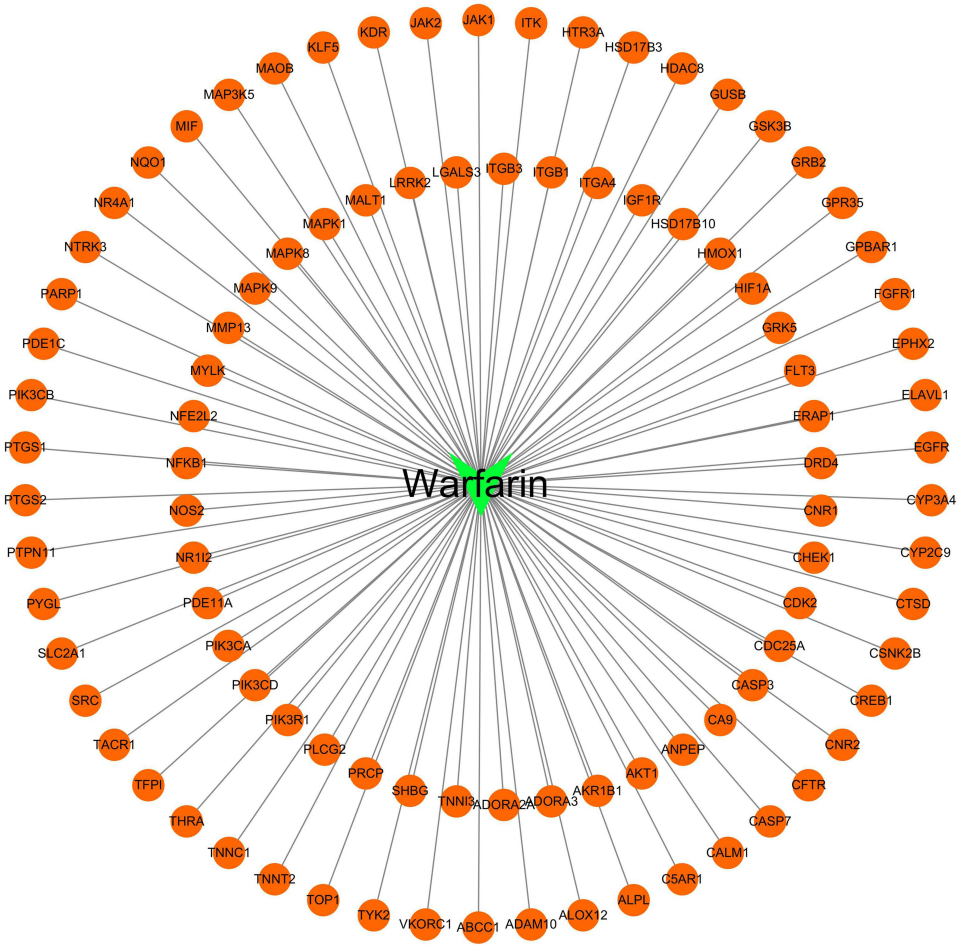

Figure 2 Target network of warfarin.

Core Targets and Network Interactions of NXT During the Coagulation Process

The 479 intersection target genes of NXT and coagulation disorders were imported into the String database for protein-protein interaction prediction. The species was set as *Homo Sapiens*, and the confidence level was set to 0.9. The targets with a degree value > 9 were selected for protein interaction network construction using the Cytoscape software. The NXT network included 139 nodes and 2046 edges. The protein-protein interaction network was constructed using degree values to reflect the size and color of targets and combined score values to reflect the thickness of edges. The results showed that TP53, AKT1, SRC, TNF, HSP90AA1, STAT3, JUN, IL6, EGFR, and ESR1 were the core targets of NXT. Moreover, MAPK9, MAP3K5, MAPK8, and MAPK1 in the MAPK family were important targets for NXT during coagulation (Figure 3).

Biological Function Enrichment

GO Analysis of Biological Processes, Cellular Components, and Molecular Functions of NXT

The intersection genes of NXT and coagulopathy were used to perform GO gene function enrichment analysis using the DAVID database. A total of 982 GO terms were screened. Using $P < 0.01$ as the standard, 715 significant biological function terms were screened for NXT in the treatment of coagulopathy. The main biological processes (BP) involved included positive regulation of gene expression, signal transduction, negative regulation of apoptosis process, inflammatory response, positive regulation of cell proliferation, response to exogenous stimulation, apoptosis process, negative regulation of gene expression, innate immune response, positive regulation of apoptosis process. There were 110 items related to cellular components (CC), involving cytosol, cytoplasm, plasma membrane, extracellular space, extracellular exosomes, extracellular region, nucleus, membrane, nucleoplasm, nucleoplasm, and mitochondria. Among them, 157

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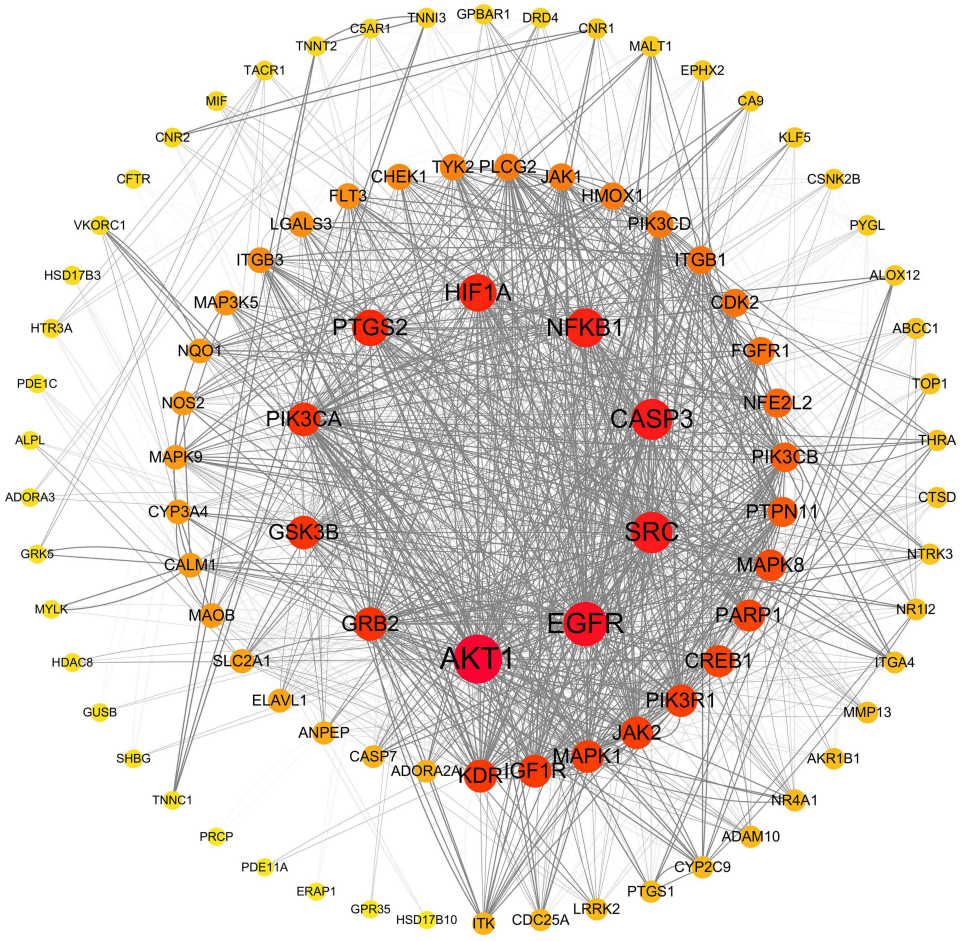

Figure 3 The core targets and network functions of NXT.

were related to molecular function (MF), involving protein binding, identical protein binding, ATP binding, enzyme binding, protein homodimerization activity, zinc ion binding, receptor binding, calcium ion binding, protein kinase binding, macromolecular complex binding (Figure 4).

KEGG Analysis of NXT Signaling Pathway

KEGG pathway enrichment analysis was performed to clarify the specific role of NXT in the signaling pathways of warfarin targets in treating coagulation disorders. Using the DAVID database, 216 pathways related to coagulation dysfunction were enriched. According to $P < 0.01$, 177 pathways related to coagulation dysfunction were screened out, and the pathways related to coagulation dysfunction were screened out. These pathways were complement and coagulation cascade, HIF-1 signaling pathway, TNF signaling pathway, PI3K-Akt signaling pathway, IL-17 signaling pathway, EGFR tyrosine kinase inhibitor resistance, FOXO signaling pathway, Toll-like receptor signaling pathway, C-type lectin receptor signaling pathway, thyroid hormone signaling pathway, Epstein-Barr virus infection, and MAP K signaling pathway, relaxin signaling pathway, T cell receptor signaling pathway, ErbB signaling pathway, VEGF signaling pathway, estrogen signaling pathway, Th17 cell differentiation, P53 signaling pathway, RAS signaling pathway, and other pathways (Figure 5).

NXT Participates in the Regulation of the Warfarin Coagulation Process by Controlling MAPK

The effect of NXT on warfarin anticoagulation was tested in rats by analyzing coagulation factors in blood before and after drug administration. In the control group (the rats were given a routine diet), no differences in Fib, TT, PT, and APTT were

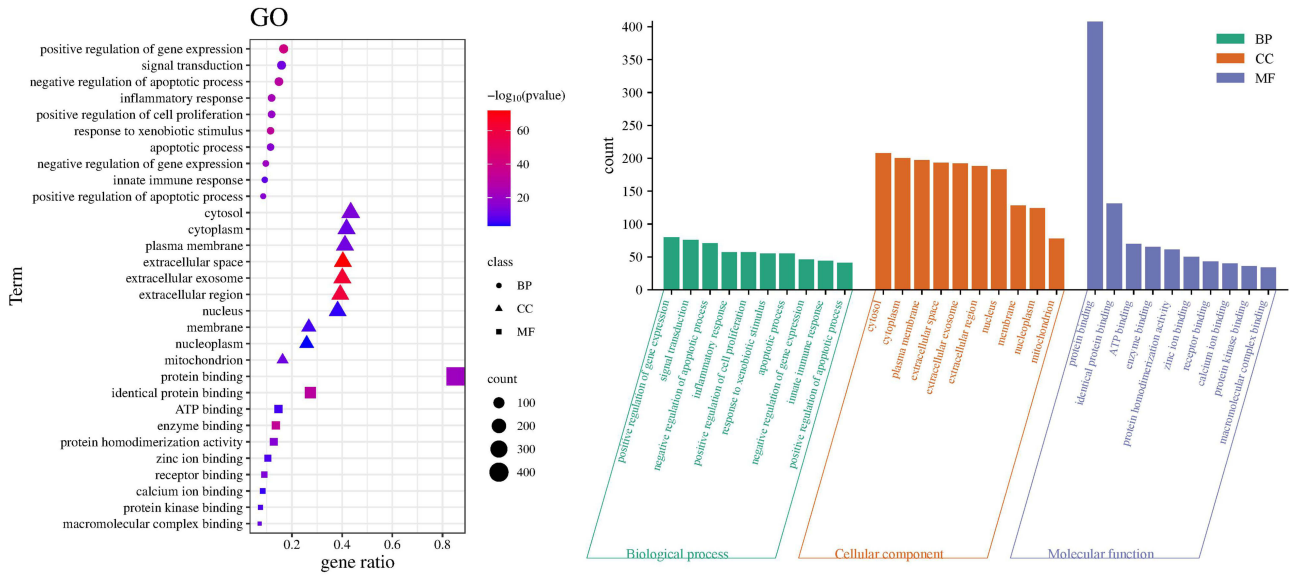

Figure 4 GO analysis of biological processes, cellular components, and molecular functions of NXT.

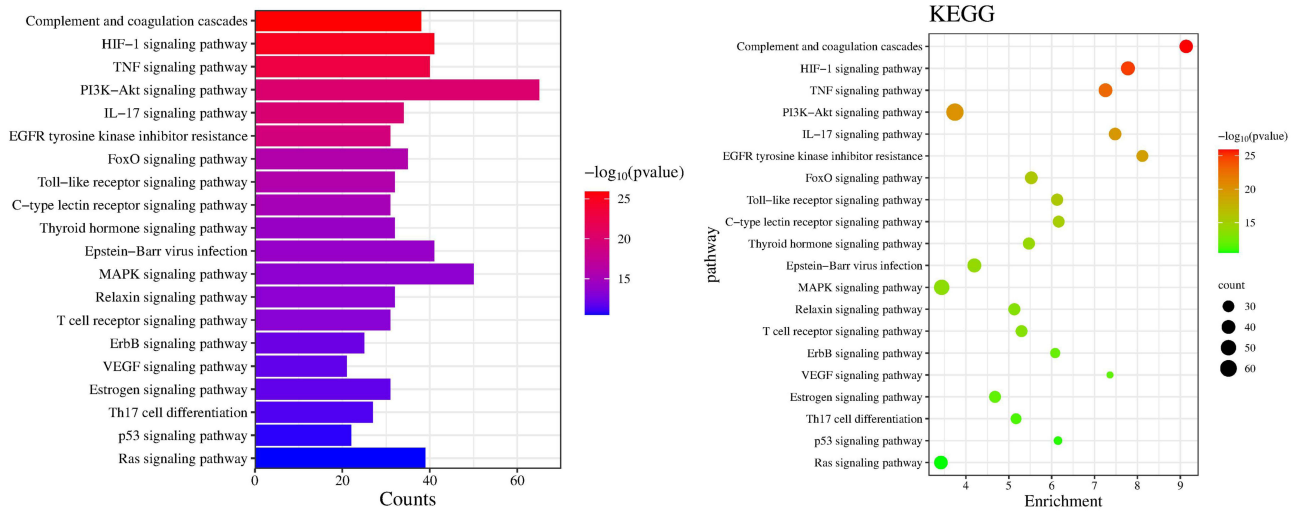

Figure 5 KEGG analysis of NXT signaling pathway. The size of the circles represents the data enriched in the genes on the corresponding pathways, green to red represents the gradually smaller p-value, and the depth of the color represents significance.

observed before and after administration (all $P > 0.05$). In the NXT group (given 1 g/kg/d NXT), there were no significant differences in Fib and APTT before and after administration (all $P > 0.05$), while TT and PT slightly decreased after administration (all $P < 0.05$). In the warfarin group (given a dose of 0.3 mg/kg/d), Fib, TT, PT, and APTT showed an increasing trend after drug administration, and the differences in the four indexes were statistically significant compared with those before medication (all $P < 0.05$). In the NXT+ warfarin group (combination group), TT, PT, and APTT showed an increasing trend after drug administration (all $P < 0.05$), no differences in Fib; yet, the PT and TT were lower than that in the warfarin group alone while no difference in Fib and APTT was found compared to the warfarin group (Figure 6).

There were no significant differences in the levels of factors II, VII, IX, and X before and after administration in the control group (all $P > 0.05$). In the NXT group, factor II showed a downward trend after administration, yet the difference was not statistically significant compared to baseline ($P > 0.05$), while factors VII, IX, and X showed an increasing trend compared to baseline (all $P < 0.05$). In the warfarin group, factors II, VII, IX, and X levels showed a downward trend after treatment (all

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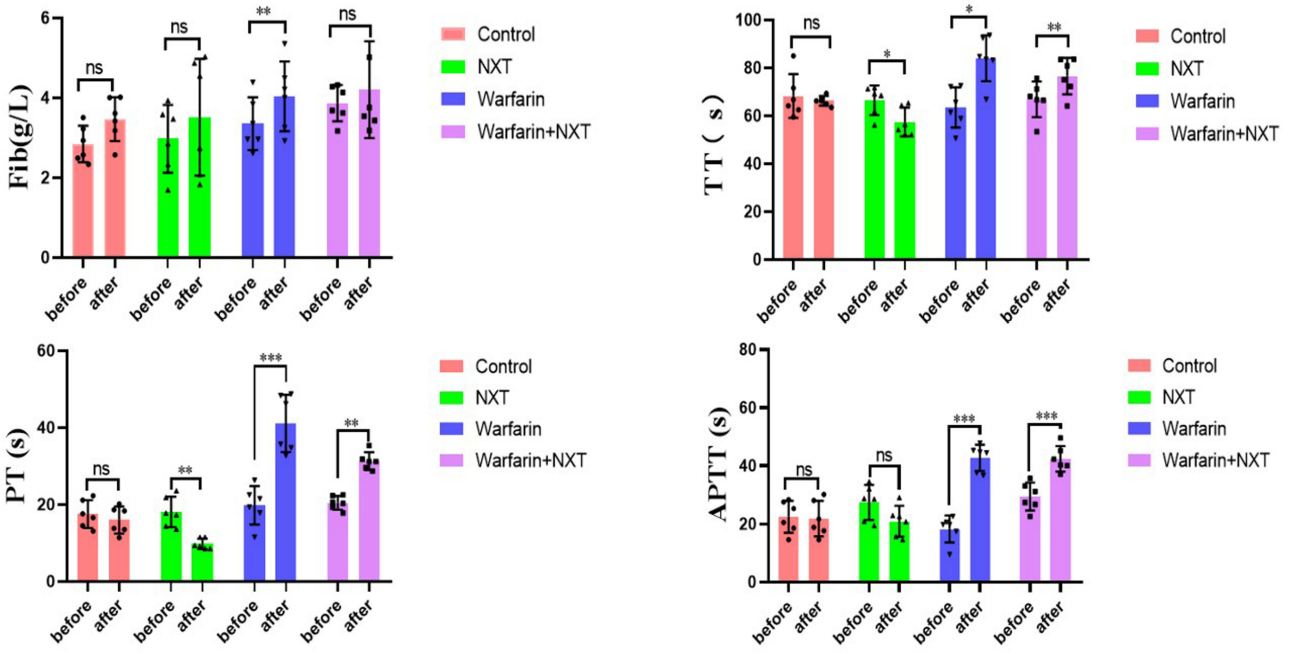

Figure 6 Comparison of four coagulation parameters (Fib, TT, PT, and APTT) before and after treatment in different groups. n=6, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.
Abbreviations: ns, not significant; Fib, Fibrinogen; aPTT, activated partial thromboplastin time; PT, prothrombin time; TT, thrombin time.

$P < 0.05$). Similar data was observed in the combined treatment group, ie, II, VII, IX, and X showed a downward trend after treatment ($P < 0.05$). However, the reduction of factors II, VII, IX, and X in the combination group was lower than in the warfarin group (Figure 7), which suggests that NXT affects warfarin therapy.

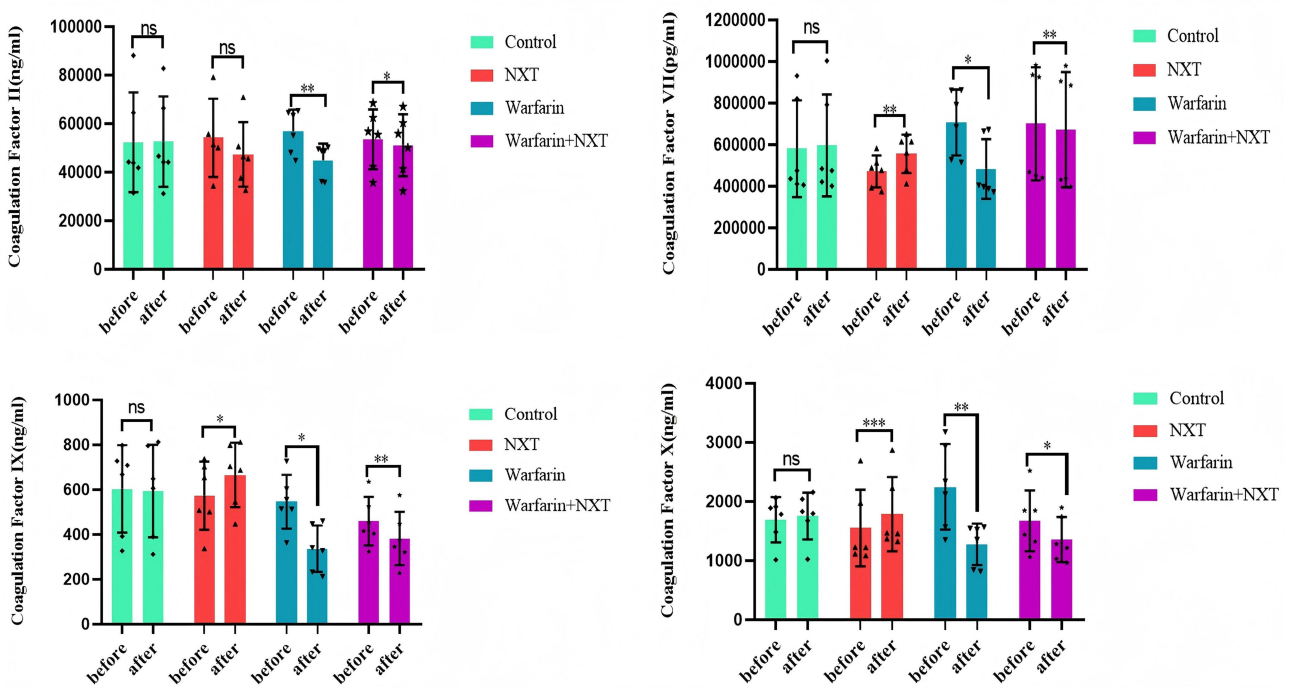

Figure 7 Comparison of coagulation factors (II, VII, IX, and (**X**) before and after treatment in different groups. n=6, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.
Abbreviation: ns, not significant.

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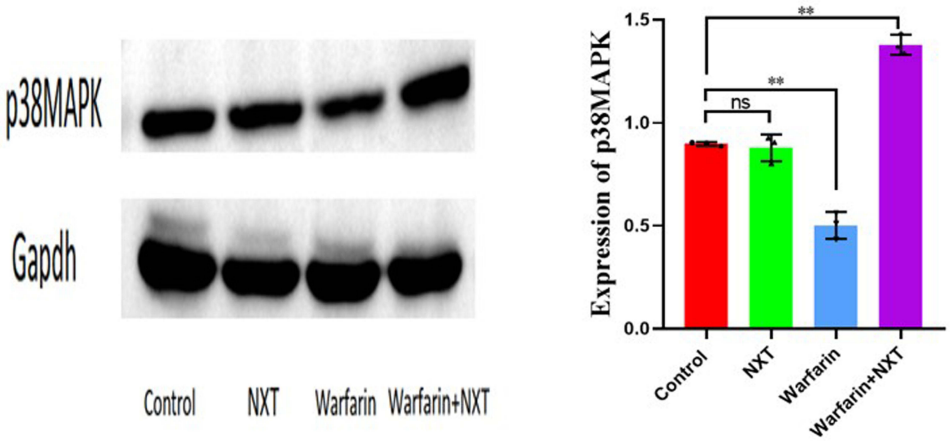

Figure 8 MAPK expression in liver tissue of rats in different groups. n=3, $^{**}P < 0.01$.
Abbreviation: ns, not significant.

Next, we used Western blot to investigate the regulation process further. Western blot results showed no significant difference in the expression of MAPK between the control group and the NTX group ($P > 0.05$), while the expression of MAPK in the warfarin group was lower than in the control group ($P < 0.01$). Compared with the control group, the expression of MAPK in the combination group showed an upward trend ($P < 0.01$). Also, the expression of MAPK in the combination group was significantly increased from that in the warfarin group ($P < 0.001$). This data implies that NTX affects warfarin therapy by regulating MAPK (Figure 8).

Discussion

In this study, we explored the effect of NXT on warfarin anticoagulation therapy and its potential mechanism. We discovered that NXT is involved in the coagulation regulation of warfarin through the MAPK pathway.

Warfarin is a drug widely used to prevent blood clots. However, it may sometimes cause serious side effects, such as heavy bleeding.[23] Furthermore, Guo et al showed that applying NXT could reduce the warfarin dose under the premise of INR reaching the target.[24] According to the 2021 Chinese atrial fibrillation guidelines, when the INR is between 3.01 and 4.00, the total weekly dose should be reduced by 10%.[25] However, this principle is difficult to achieve in real-world clinical management because 10% of the total drug dose is difficult to control, and the drug dispensing process is impossible to achieve.[26] A recent follow-up study on the anticoagulant benefit management of warfarin in atrial fibrillation indicated that applying NXT could prolong the INR target time, which means an increase in the number of INR targets within a specific time range.[27]

Screening coagulation assays are usually based on examining PT, INR, APTT, and TT. The thrombin clot time can be further modified to measure Fib concentration.[28] In this study, we further tested the effect of NTX on coagulation parameters (PT, APTT, Fib, TT) in rats given warfarin. A combination of NXT plus warfarin increased Fib, TT, PT, and APTT; yet, the PT and TT were lower than that in the warfarin group alone, while no difference in Fib and APTT was found compared to the warfarin group. TT, also known as the thrombin clotting time, is a blood test that measures the time it takes for a fibrin clot to form in the plasma, while PT is a test that measures the time it takes for the liquid portion (plasma) of blood to clot. TT is also mainly used to assess the activity status of coagulation factors I, II, V, VIII, X, and XIII in plasma. In this study, we found a decrease in II, VII, IX, and X factors after treatment in both warfarin and combination groups (all $P < 0.05$ vs control); however, the reduction of factors II, VII, IX, and X in the combination group was lower than in the warfarin group (Figure 7), which suggests that NXT affects warfarin therapy.

Studies have shown that quercetin, kaempferol, luteolin, and sitosterol in prescription drugs are the main material bases for NXT to participate in coagulation.[29] Xie et al[30] confirmed that tanshinone and kaempferol can improve the "blood stasis" state of the body and maintain the stability of hemodynamics, thereby reducing thrombotic events. The results of this study showed that the target genes of NXT are mainly enriched in phosphatidylinositol 3-kinase (PI3K) - protein kinase B (Akt) and adenosine monophosphate-activated protein kinase (MAPK) pathways. Many members of the

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MAPK family are important targets for warfarin to exert an anticoagulant effect.[31,32] Li et al[32] pointed out that the activation of the ERK/MAPK pathway is closely related to the coagulation process. However, whether the MAPK pathway is involved in regulating warfarin metabolic enzymes is still unknown. Based on pharmacological theory and some validated preliminary studies, we further explored whether NXT affects the specific action process of warfarin through the MAPK signaling pathway. Our results showed that NTX participates in the anticoagulation process of warfarin through up-regulation of MAPK and mainly exerts an antagonistic effect.

The present study showed that NXT has a potential bidirectional regulatory effect on the effects of warfarin. Therefore, when elaborating a clinical treatment plan for a specific patient, physicians may consider reducing the dosage of warfarin when other factors present may cause NXT to activate the thrombotic process of warfarin. On the other hand, when conditions that could inhibit the anticoagulant effect of warfarin are present, eg, by affecting the metabolic pathway of the NXT effect, the dose of warfarin may need to be increased to maintain the intensity and effectiveness of warfarin therapy. Nevertheless, additional studies are necessary to confirm these views. NXT is a drug clearly mentioned in the instruction manual of common drug prescriptions for cardiovascular diseases that can affect coagulation. Meanwhile, compared with Shexiang Baoxin pills[33–35] and Qishen Yiqi dropping pills,[36,37] it has a wider range of clinical indications. Therefore, the present study focused on the NXT TCM preparation, but other preparations should also be investigated in the future.

This study has a few limitations. Due to the relatively small number of samples selected in this study, sample heterogeneity and selection bias inevitably may exist. Also, no thrombosis model was constructed in this study. Therefore, the results of this study can only indicate that NXT may affect the action of warfarin and participate in the coagulation process through the MAPK signaling pathway. However, whether this conclusion applies to different disease models and deeper mechanism exploration needs to be further verified with more data.

Conclusion

To conclude, our data show that NXT affects the coagulation factors when taking warfarin, and it may have a bidirectional regulation effect on coagulation factors (NXT can shorten PT and APTT in warfarin patients, and reduce coagulation factors II but increase VII, IX, and X). Also, NXT can participate in regulating the coagulation process of warfarin through multiple targets and pathways, especially MAPK.

Data Sharing Statement

All data generated or analyzed during this study are included in this article.

Ethics Approval and Consent to Participate

All animal studies (including the mice euthanasia procedure) were done in compliance with the regulations and guidelines of Nanchang University institutional animal care and conducted according to the AAALAC and the IACUC guidelines (Ethical review number: JJSDYRMYY-YXLL-2023-130).

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis, and interpretation, or in all these areas; took part in drafting, revising, or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors declare that they have no competing interests.

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