Journal of Ginseng Research 48 (2024) 494–503

Contents lists available at ScienceDirect

Journal of Ginseng Research

journal homepage: www.sciencedirect.com/journal/journal-of-ginseng-research

Research Article

Mechanism of Panax notoginseng saponins modulation of miR-214-3p/NR1I3 affecting the pharmacodynamics and pharmacokinetics of warfarin

Yuting Yang [a,1], Zhenyu Zhai [b,1], Huiming Yao [a], Ling He [c], Jun Shao [d], Zirong Xia [b,**], Juxiang Li [b,*]

[a] Department of Critical Care Medicine, Medical Center of Anesthesiology and Pain, The First Affiliated Hospital, Jiangxi Medical College, Nanchang University, China
[b] Department of Cardiovascular Medicine, The Second Affiliated Hospital of Nanchang University, Nanchang, China
[c] Department of Gerontology, The Second Affiliated Hospital of Nanchang University, Nanchang, China
[d] Department of Cardiac Surgery, The Second Affiliated Hospital of Nanchang University, Nanchang, China

ARTICLE INFO

Keywords:
Panax *notoginseng saponins*
Warfarin
Pharmacokinetics
miR-214-3p
NR1I3

ABSTRACT

Background: With the prevalence of dietary supplements, the use of combinations of herbs and drugs is gradually increasing, together with the risk of drug interactions. In our clinical work, we unexpectedly found that the combination of Panax notoginseng and warfarin, which are herbs that activate blood circulation and remove blood stasis, showed antagonistic effects instead. The purpose of this study was to evaluate the drug interaction between Panax *notoginseng saponins* (PNS) and warfarin, the main active ingredient of Panax notoginseng, and to explore the interaction mechanism.

Methods: The effects and mechanisms of PNS on the pharmacodynamics and pharmacokinetics of warfarin were explored mainly in Sprague–Dawley rats and HepG2 cells. Elisa was used to detect the concentrations of coagulation factors, HPLC-MS to detect the blood concentrations of warfarin in rats, immunoblotting was employed to examine protein levels, qRT-PCR to detect mRNA levels, cellular immunofluorescence to detect the localization of NR1I3, and dual luciferase to verify the binding of miR-214-3p and NR1I3.

Results: PNS significantly accelerated warfarin metabolism and reduced its efficacy, accompanied by increased expression of NR1I3 and CYP2C9. Interference with NR1I3 rescued the accelerated metabolism of warfarin induce by PNS co-administration. In addition, we demonstrated that PNS significantly reduced miR-214-3p expression, whereas miR-214-3p overexpression reduced NR1I3 and CYP2C9 expression, resulting in a weakened antagonistic effect of PNS on warfarin. Additionally, we found that miR-214-3p bound directly to NR1I3 3′-UTR and significantly downregulated NR1I3 expression.

Conclusion: Our study demonstrated that PNS accelerates warfarin metabolism and reduces its pharmacodynamics by downregulating miR-214-3p, leading to increased expression of its target gene NR1I3, these findings provide new insights for clinical drug applications to avoid adverse effects.

1. Introduction

Warfarin is the most widely used anticoagulant drug in the world and has been clinically used for over 50 years, primarily in the prevention and treatment of atrial fibrillation, valve disease, prosthetic valve replacement and intracardiac thrombosis, and venous thromboembolic disease [1]. However, warfarin has a narrow therapeutic window, is sensitive to individual differences, is influenced by drugs or food, has significant drug interactions, and requires efficacy monitoring based on the international normalized ratio [2]. In recent years, the combination of herbal medicine has become increasingly prominent in terms of adverse effects and has attracted the attention of researchers. In our clinical work, we found that Panax notoginseng can significantly reduce the INR value of warfarin, and rat experiments confirmed that PNS can

* Corresponding author. Department of Cardiovascular Medicine The Second Affiliated Hospital of Nanchang University, Minde Road No.1, Nanchang, Jiangxi, 330006, China.
** Corresponding author. Department of Cardiovascular Medicine, The Second Affiliated Hospital of Nanchang University, Nanchang, 330006, China.
E-mail addresses: ziwan0405@hotmail.com (Y. Yang), 13337107502@163.com (Z. Zhai), yao.huiming@163.com (H. Yao), ndefy22058@ncu.edu.cn (L. He), 601476619@qq.com (J. Shao), 425599293@qq.com (Z. Xia), ljx912@126.com (J. Li).
[1] These authors contributed equally: Yuting Yang and Zhenyu Zhai.

https://doi.org/10.1016/j.jgr.2024.05.003
Received 1 April 2024; Received in revised form 14 May 2024; Accepted 14 May 2024
Available online 18 May 2024

accelerate the metabolism of warfarin and reduce the efficacy of the drug. Therefore, it is of clinical significance to further investigate the effect of Panax notoginseng on the pharmacodynamics and pharmacokinetics of warfarin and its mechanism for the management of anticoagulated patients.

Panax notoginseng is the dried root and rhizome of Panax notoginseng (Burk.) F.H.Chen], a plant of the family Wujia family, and is one of the most commonly used herbs, known for its efficacy in activating blood circulation, resolving blood stasis, and relieving swelling and pain. Panax *notoginseng saponins* (PNS) are the main active ingredients of Panax notoginseng, and are mainly composed of dammarane type 20(S)-ginseng diol (protopanaxadiol, PPD), protopanaxatriol (PPT) [3]. Studies have shown that PNS FT- 1 can prolong tail bleeding time in mice, probably by antagonizing various agonists that induce platelet aggregation (e.g., collagen, thrombin, and adenosine diphosphate), thereby inhibiting intracellular calcium mobilization, reducing phosphorylation of ERK and p38, blunting ERK/p38 activation of platelet aggregation, and prolonging bleeding time. Another study found that PNS significantly shortende the prolonged bleeding time caused by aspirin [4]. Thus, Panax ginseng may exert bidirectional regulatory effect on coagulation.

NR1I3 (constitutive androstane receptor, CAR) is a member of the nuclear receptor superfamily and plays an important role in coordinating cellular responses to endogenous and exogenous chemical stimuli by regulating the expression of target genes [5]. Un-activated NR1I3 is present in the phosphorylated form in the cytoplasm, protein phosphatase 2Ac uses the protein kinase 1 receptor as a regulatory subunit to dephosphorylate Thr38 to activate NR1I3, which after activation enters the nucleus and regulates the expression of downstream target genes [6]. NR1I3 mainly induces the CYP2B family of phase I metabolic enzymes and the phase II metabolic enzyme UGT1A1, which are essential for drug delivery in mice [7]. NR1I3 promotes the transcriptional translation of CYP2C9 by binding to transcriptional regulatory elements of CYP2C9 gene flanking sequences and thus affecting drug metabolism [6]. These studies suggest that NR1I3 may play a key role in the effect of PNS on the pharmacodynamics and pharmacokinetics of warfarin.

Previous studies have shown that upstream microRNAs (miRNAs) can regulate the mRNA levels of target genes. Thus, identifying target gene-related miRNAs is research topic. In recent years, with the continuous discovery and in-depth study of miRNAs, which opens a new field for the study of drug metabolism and drug interaction mechanism, the relationship between miRNAs and drug metabolism has become a hot topic for researchers in related disciplines. Deng et al. [8] found that the long non-coding RNA(lncRNA) F11-AS1 regulates miR-211-5p by binding to NR1I3, thereby inhibiting HBV-associated hepatocellular carcinoma. Wang et al. [9] found that lncRNA HNF1-AS1 was positively correlated with NR1I3 expression by lncRNA microarray analysis of human liver tissue, confirming that lncRNA HNF1-AS1 affects CYP450 family protein expression through activation of NR1I3. These studies suggest that miRNAs play a crucial role in regulating NR1I3, thereby affecting drug metabolism. However, it is unclear whether post-transcriptional regulation by miRNAs is also involved in the effect of PNS on the pharmacodynamics and pharmacokinetics of warfarin. Therefore, the objective of this study was to investigate the drug interactions between PNS and warfarin and to explore the potential mechanisms.

2. Materials and methods

2.1. Reagents

PNS with purity >98 %, as determined by HPLC were purchased from Solarbio Technology Corporation (Beijing, China). The chemical structures and HPLC-UV chromatographic fingerprints of the main components of the PNS are shown in Supplementary Fig. 1. Warfarin was obtained from the Shanghai Acmec Biochemical Co. (Shanghai, China). The cell counting kit-8 was purchased from Beyotime Biotechnology (Shanghai, China). Elisa kits were purchased from Shanghai Enzyme Link Biotechnology Co.

2.2. Cell culture

HepG2 cells were obtained from American Type Culture Collection (Manassas, VA, USA). HepG2 cells were cultured and passaged in DMEM containing 10 % fetal bovine serum at 5 % CO2 and maintained at 37 °C. HepG2 cells were stimulated with 50 μg/mL of PNS for 24 h and(or) 50 μM of warfarin for 24 h, cellular proteins and mRNA were extracted for detection.

2.3. Animal experiments

Six to eight weeks old male Sprague-Dawley rats, from the Model Animal Research Center of Wuhan University, were acclimatized and fed for one week and then administered the PNS of 200 mg/kg/d and warfarin dose of 0.3 mg/kg/d. The control group was administered 0.5 % sodium carboxymethylcellulose and the rats in the groups were continuously gastric gavaged continuously for one week. At 0.5, 1, 2, 3, 4, 6, 8, 10, 12, 16, 20, 24, 48h after the last dose, 500 μL of blood was collected from the inner canthus vein of rats by centrifugation at 3000 rpm for 15min, and the supernatant was stored at −20 °C. Simultaneously, blood was collected from the internal canthus vein into an anticoagulation tube at the 12h after the last administration, and the upper plasma was collected for coagulation function and coagulation factor II and X assays. The animal experiments were approved by the animal protection and utilization committee and complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978).

2.4. Detection of warfarin concentration and calculation of pharmacokinetic parameters

We developed an HPLC-MS/MS method to detect warfarin drug concentrations. The analysis was performed on a Voyager TSQ quantum triple quadrupole instrument equipped with an Ultimate 3000 Chromatography system (Thermo Fisher Scientific, San Jose, California, USA) in positive ion mode. A Welch Ultimate UHPLC XB-C18 column (50 × 2.1 mm, 1.8 μm) was used to separate the components. The mobile phase was formic acid-water containing 0.1 % formic acid (90:10, v/v) at a flow rate of 0.4 mL/min. Chromatograms of analytes and internal standards were acquired and integrated by the software of Xcalibur 3.0 (Thermo Fisher Scientific), and linear regression was performed with a weighting factor of 1/X2.

All obtained plasma were stored at −80 °C until HPLC-MS/MS analysis. Pharmacokinetic parameters, including area under the curve (AUC), mean retention time (MRT), apparent volume of distribution (Vz/F), clearance (CLz/F), peak concentration (Cmax), time to peak (Tmax), and half-life (t1/2) were calculated using WinNonlin 5.2.1 pharmacokinetic software based on the administered dose and the corresponding blood concentrations of warfarin at each time point in rats.

2.5. Quantitative real-time polymerase chain reaction and Western blot analysis

Total RNA from liver tissues and HepG2 cells was isolated using TRIzol Universal (Tiangen DP424). Quantitative analysis of target mRNA expression was performed by qRT-PCR using the relative standard curve method. TaqMan and SYBR Green analyses were conducted using an Applied Biosystems 7500 Sequence Detector (Applied Biosystems), and GAPDH gene expression was used as the standard. All primers use for qRT-PCR are listed in Supplemental materials. Western blot analysis for the expression of specific protein was performed by the standard method. The antibodies used were anti-NR1I3 (1:1000, Abcam

cat: ab186869, UK), anti-CYP2C9 (1:1000, Abcam cat: ab150364, UK), anti-VKORC1 (1:1000, Abcam cat: ab206656, UK), anti-CYP3A4 (1:2000, Proteintech cat:18227-1-AP, China), anti-CYP1A2(1:3000, Proteintech cat:19936-1-AP, China) and anti-Tubulin (1:5000, Proteintech cat: 11224-1-AP China). Signal detection was performing by enhanced chemiluminescence (USA).

2.6. Immunofluorescence

HepG2 cells were inoculated in 48-well plates and immobilized in 4 % paraformaldehyde at room temperature for 1 h. The immobilized cells were permeabilized with 0.1 % Triton-X100 for 30 min and then blocked with 10 % BSA or 10 % goat serum for 30 min at room temperature. A 1:100 dilution of the primary antibody was used and an appropriate volume was added to the corresponding wells and incubated overnight at 4 °C. After washing, add fluorescent secondary antibody (1ug/ml, Thermofisher) and incubate for 2 h, then stained with DAPI (1:1000, Solarbio, C0060 CHINA). Images were detected by fluorescence microscopy.

2.7. Statistical analysis

All results are shown as the mean ± SD and were analyzed from at least three independent experiments using GraphPad Prism 8 (GraphPad software, USA). Two-tailed unpaired Student's *t*-test was used for analyzing the comparison between the two groups and one-way ANOVA was used for analyzing the comparison among multiple groups. $P < 0.05$ was regarded as statistically. Error bars represent SEM, ns: no significant difference; *$P < 0.05$; * *$P < 0.01$; * * *$P < 0.001$.

3. Results

3.1. PNS accelerates the drug metabolism of warfarin and reduces pharmacodynamics

To investigate the effect of PNS on the efficacy of warfarin, we first tested the coagulation function of rats. The results showed that the INR and APTT were significantly increased in the warfarin group compared with the control group. However, when PNS and warfarin were combined, the elevated INR and APTT caused by warfarin were restored (Fig. 1A and B). Meanwhile, warfarin could reduce plasma coagulation factors II and X. PNS had no significant effect on coagulation factors, but it could rescue the decrease of coagulation factors caused by warfarin (Fig. 1C and D).

Next, we assayed the blood concentration of warfarin. After the rats were administered in experimental groups, blood samples were collected at points according to the protocol and the plasma warfarin concentration in each group was measured using the established HPLC-MS/MS method. The drug concentration-time curves showed that PNS accelerated the metabolism of warfarin in rats (Fig. 1E). The results of pharmacokinetic parameters showed that the AUC and MRT decreased, and the Vz/F and CLz/F increased in the combination group compared to those in the warfarin group alone, while the differences in Cmax, Tmax and t1/2 were not statistically significant (Supplemental Table 1). Thus, PNS reduced the pharmacodynamics of warfarin by accelerating its metabolism in rats.

Next, cellular experiments were performed, HepG2 cells were drug-stimulated to detect changes in coagulation factor II and X concentrations using an Elisa kit. The results showed that PNS alone had no significant effect on coagulation factors, but PNS could significantly reduce the inhibitory effect of warfarin on coagulation II and X, and the difference was statistically significant (Fig. 1F and G). The above results suggest that PNS can still reduce the efficacy of warfarin in HepG2 cells. Collectively, these data indicate that PNS accelerated warfarin metabolism and reduced its pharmacodynamics.

3.2. PNS stimulation increases the expression of NR1I3

It is known that warfarin is mainly metabolized in vivo by hepatic drug metabolizing enzymes CYP2C9, CYP1A2 and CYP3A4, and the target of drug action is vitamin K epoxide reductase complex (VKORC) [10]. Therefore, we examined the expression levels of these proteins. The results showed that CYP2C9 increased significantly after PNS stimulation, while the levels of other drug-metabolizing enzymes CYP1A2, CYP3A4 and the target VKORC1 protein and mRNA did not change significantly (Fig. 2A–D), suggesting that PNS accelerates the

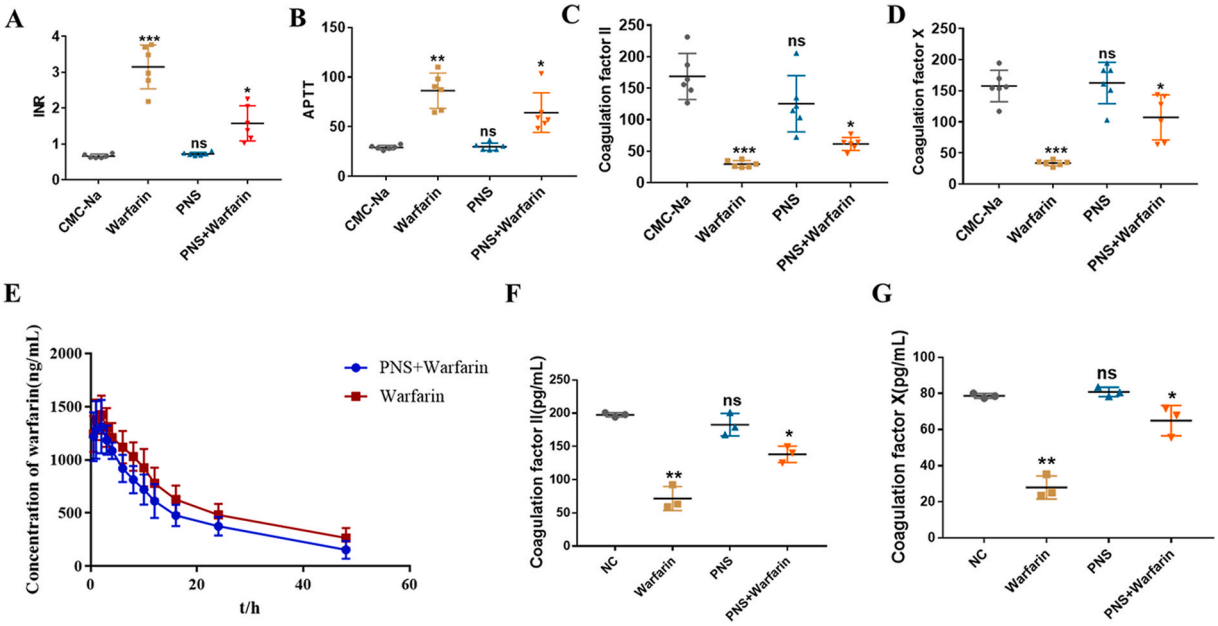

Fig. 1. (A: The effect of PNS on INR value; B: The influence of PNS on APTT value; C: The effect of PNS on coagulation factor II; D: The effect of PNS on coagulation factor X; E: The concentration time curve of rat plasma, the horizontal coordinate is the time since the last administration, and the vertical coordinate is the concentration of rat plasma, n = 5; F: The effect of PNS on coagulation factor II in HepG2 cells; G: The effect of PNS on coagulation factor X in HepG2.)

drug metabolism of warfarin probably by inducing CYP2C9, the main drug-metabolizing enzyme of warfarin.

To investigate the reason for the increase of CYP2C9 expression, we examined its transcription factor NR1I3. Immunohistochemical analysis of rat liver tissues suggested that the expression of CYP2C9 and NR1I3 expression levels increased after PNS stimulation (Fig. 2E and F). Meanwhile, Western blotting and qRT-PCR results showed that the protein and mRNA levels of NR1I3 were elevated in rat liver after PNS treatment (Fig. 2G and H). In addition, we validated this finding in HepG2 cells. The protein and mRNA levels of CYP2C9 and NR1I3 were upregulated in HepG2 cells after PNS treatment (Fig. 2I–K). Therefore, we speculated that the effect of PNS on warfarin efficacy may be mediated by the induction of drug metabolizing enzyme CYP2C9 by NR1I3 and accelerated warfarin drug metabolism.

3.3. PNS affects the pharmacodynamics and pharmacokinetics of warfarin through the induction of NR1I3 in HepG2 cells

Since NR1I3 has an activating effect on CYP2C9, the main drug metabolizing enzyme of warfarin, whether NR1I3 is a key factor in the process of PNS affecting the efficacy and pharmacokinetics of warfarin requires further study. First, we generated a stable cell line of HepG2 in total. The protein and mRNA expression of sh-NR1I3#1 in the four interfering sequences were significantly lower than those of the control group, and the protein and mRNA expression of overexpressed NR1I3 were significantly higher than those of the control group (Fig. 3A–D), which proved that the stable cell line was successfully constructed. We next performed pharmacological stimulation, and the results suggested that the restoring effect of PNS on coagulation factors was reduced after knockdown of NR1I3 compared with the control group, and coagulation factor II and coagulation factor X showed the same trend of change (Fig. 3E and F). Meanwhile, the salvage effect of PNS on coagulation factors was further enhanced after the overexpression of NR1I3 (Fig. 3G

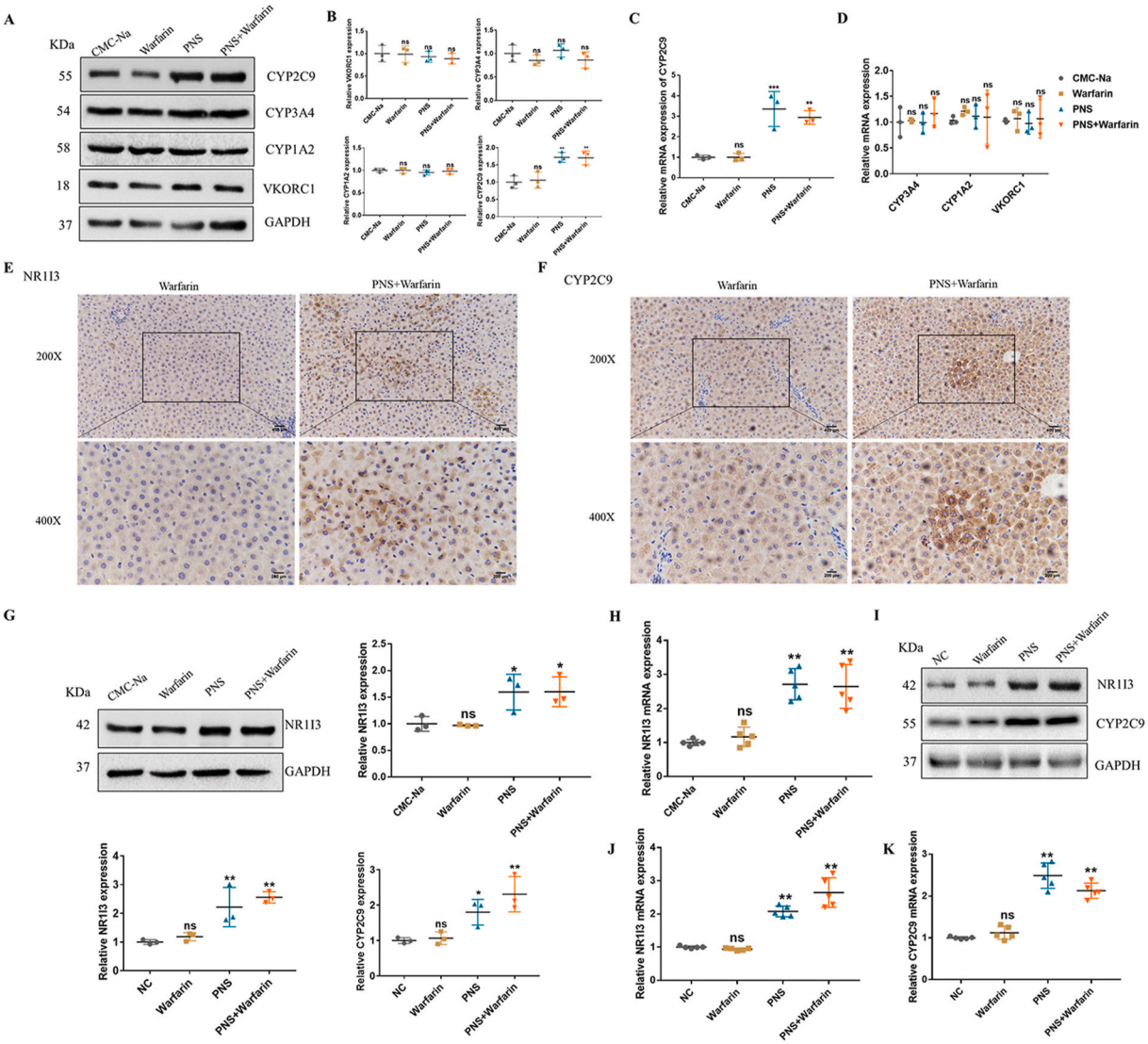

Fig. 2. (A: Changes in protein levels of drug metabolizing enzymes in rat liver; B: Statistical graph of changes in protein levels of drug metabolizing enzymes in rat liver; C: Changes in transcript levels of CYP2C9 in rat liver; D: Changes in transcript levels of drug metabolizing enzymes in rat liver, n = 3; E,F: Immunohistochemical detection of the effect of PNS on the expression of NR1I3 and CYP2C9 in rat liver, the scale bar is 200um,the upper line is 200X,the lower line is 400X as shown in the upper line box; G: the effect of PNS on the protein of NR1I3 in the rat liver and the statistical graphs; H: the effect of PNS on the level of transcription of NR1I3 in the rat liver; I: Effect of PNS on NR1I3 and CYP2C9 protein expression in HepG2 cells and statistical graphs; J, K: Effect of PNS on NR1I3 and CYP2C9 transcript levels in HepG2 cells and statistical graphs.)

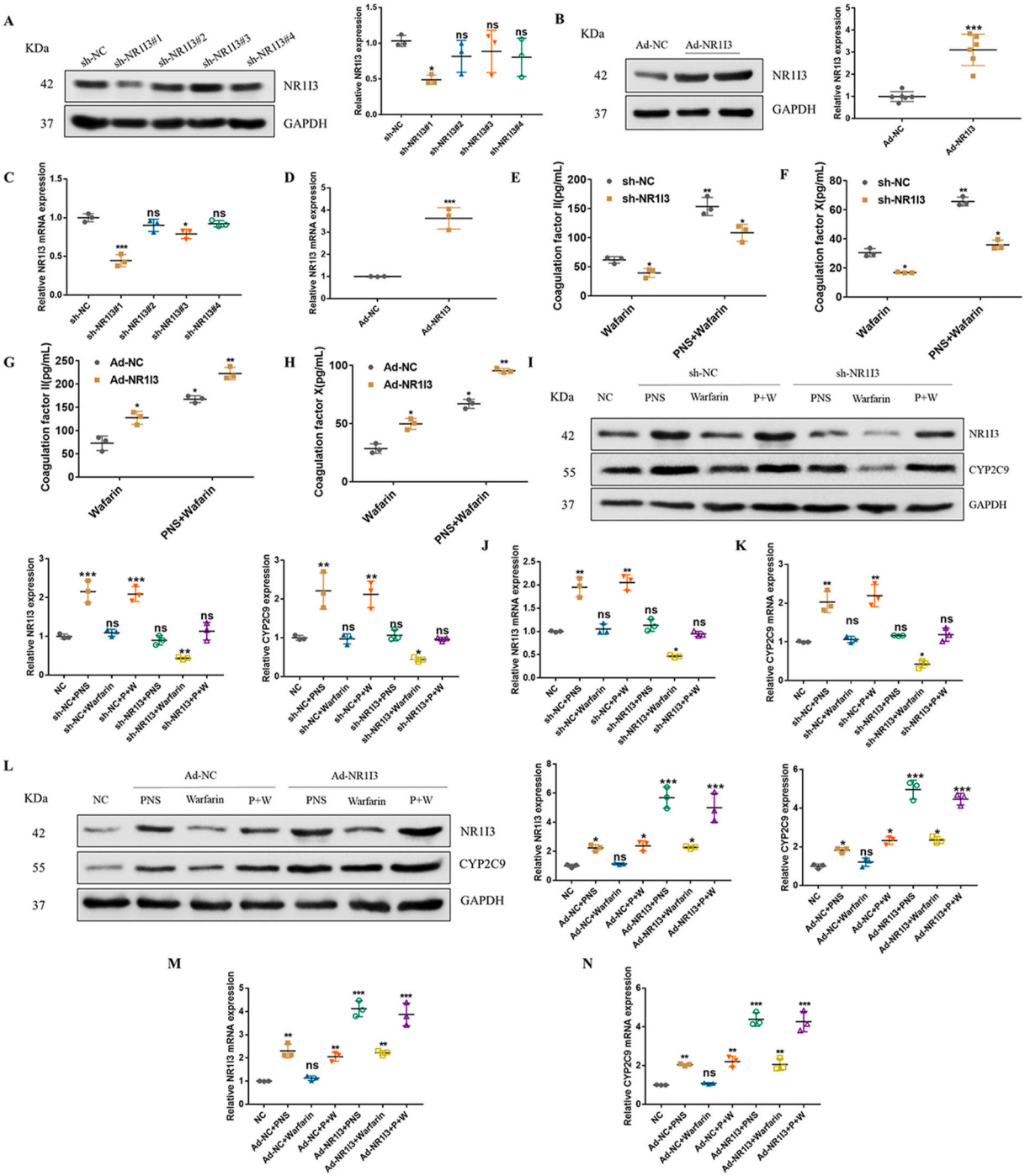

Fig. 3. (A: Validation of interference with NR1I3 at the protein level; B: Validation of overexpression of NR1I3 at the protein level; C: Validation of interference with NR1I3 at the transcriptional level; D: Validation of overexpression of NR1I3 at the transcriptional level; E, F: Changes in coagulation factors II and X after interference with NR1I3, n = 3; G, H: Changes in coagulation factors II and X after overexpression of NR1I3, compared with the sh-NC + Warfarin group and the Ad-NC + Warfarin group, n = 3; I: Protein expression and statistics graphs of NR1I3 and CYP2C9 after interference NR1I3 and dosing treatment; J: Changes in NR1I3 mRNA expression after interference NR1I3 and dosing treatment; K: Changes in CYP2C9 mRNA expression after interference NR1I3 and dosing treatment; L: Protein expression and statistics graphs of NR1I3 and CYP2C9 after overexpression of NR1I3 and dosing treatment; M: Changes in NR1I3 mRNA expression after overexpression of NR1I3 and dosing treatment; N: Changes in CYP2C9 mRNA expression after overexpression of NR1I3 and dosing treatment.)

and H).

To further verify the regulatory mechanism of NR1I3 in the interaction between PNS and warfarin, we examined the changes of NR1I3 and CYP2C9 genes. NR1I3 and CYP2C9 protein and mRNA levels were upregulated after PNS stimulation, and when NR1I3 was interfered, the protein and mRNA levels of CYP2C9 were subsequently downregulated, while the elevated expression of NR1I3 and CYP2C9 due to PNS was reverted (Fig. 3I–K). Overexpression of NR1I3 was followed by upregulation of protein and mRNA levels of CYP2C9, while the elevated expression of NR1I3 and CYP2C9 due to PNS was more significant (Fig. 3L–N). These results demonstrate that NR1I3 is a key factor in the drug interaction between PNS and warfarin in in vitro experiments.

3.4. PNS affects the pharmacodynamics and pharmacokinetics of warfarin by inducing NR1I3 in rats

Further animal experiments were conducted to demonstrate whether NR1I3 remains a key gene in the drug interaction between PNS and warfarin in rats. Rats were treated with adeno-associated virus via tail vein injection followed by gavage, and relevant specimens were collected for testing. The results showed that the effect of PNS on reducing warfarin INR and APTT was decreased after NR1I3 knockdown compared with the control group, and the effect of PNS in reverting to coagulation factors was correspondingly decreased (Fig. 4A–D). In contrast, the effect of PNS on reducing warfarin INR and APTT was more significant after overexpression of NR1I3, and the effect of PNS in rescuing coagulation factors was increased accordingly (Fig. 4E–H).

Subsequently we examined the blood concentration of warfarin in each group of rats. The results of drug concentration-time curves showed that PNS could accelerate the metabolism of warfarin in rats, and knockdown of NR1I3 down-regulated the accelerated metabolism of warfarin caused by PNS(Fig. 4I). The results of the pharmacokinetic parameters (Supplemental Table 2) showed that the decreasing trend of the AUC caused by PNS disappeared, and the increasing trend of Vz/F and CLz/F were eliminated with statistically significant differences, while the differences of MRT, Tmax and t1/2 were not statistically significant. Meanwhile, the results of drug concentration-time curves after overexpression of NR1I3 in rats showed that PNS accelerated the metabolism of warfarin in rats, and overexpression of NR1I3 could exacerbate the accelerated metabolism of warfarin caused by PNS (Fig. 4J). The results of pharmacokinetic parameters (Supplemental Table 3) showed that overexpression of NR1I3 led to a more significant decrease in the AUC and MRT, and an increase in the Vz/F and CLz/F, with statistically significant differences, while the differences of Tmax and t1/2 were not statistically significant.

The liver tissues of rats in each group were collected, and changes of NR1I3 and CYP2C9 genes were detected. After knockdown of NR1I3, NR1I3 and CYP2C9 protein and mRNA levels were significantly down-regulated, indicating successful viral transfection. Meanwhile, the elevated NR1I3 and CYP2C9 protein levels caused by PNS decreased, and the mRNA and protein levels trended in agreement (Fig. 4K-M). In addition, after the overexpression of NR1I3, the elevated levels of NR1I3 and CYP2C9 proteins caused by PNS were more significant (Fig. 4N–P). The above experimental results further demonstrate that NR1I3 is a key molecule in the interaction between PNS and warfarin in in vivo experiments, and acts through the regulation of CYP2C9, the main drug metabolizing enzyme of warfarin.

3.5. miR-214-3p is a key determinant in the drug interaction between PNS and warfarin

With increasing research on miRNAs, the reports of miRNA regulation by active ingredients of herbal medicines are gradually increasing. Nuclear receptors play an important role in cellular responses to environmental stimuli by activating or repressing the expression of genes, including those encoding drug transporters and biotransformation enzymes. This type of interaction increases the complexity of miRNAs in regulating the role of drug-metabolizing enzymes, and our previous study demonstrated that the heterologous nuclear receptor NR1I3 regulates the role of drug-metabolizing enzyme CYP2C9, but whether miRNAs are involved in this process needs to be confirmed by further studies. The TargetScan online prediction software was used to predict miRNAs that may interact with NR1I3. Based on the pairing mode, context++ score percentile and literature survey, four miRNAs that might act with NR1I3 were screened: miR-214-3p, miR-125a-3p, miR-25-3p and miR-155-5p. Next, HepG2 cells were treated with drug to extract cellular miRNAs and detect the expression abundance of the above four miRNAs. The results of qRT-PCR showed that compared with the warfarin group, only the expression of miR-214-3p decreased (Fig. 5A). Additionally, miRNAs from rat liver tissues were extracted and the expression was examined, qRT-PCR results were consistent with cells (Fig. 5B). Therefore, miR-214-3p may be a key miRNA that regulate the drug interactions between PNS and warfarin.

To further verify the direct binding of miR-214-3p and NR1I3-3′UTR, a luciferase reporter gene assay was used. The results showed that overexpression of miR-214-3p significantly inhibited the reporter gene luciferase activity compared to the control, and the difference was statistically significant. However, there was no significant difference in the effect of overexpression of miR-214-3p on the luciferase activity of the reporter gene after the mutation of the binding site. Taken together, the above experimental results demonstrated that miR-214-3p and the 3′UTR of NR1I3 mRNA could bind directly (Fig. 5C).

Next, we constructed miR-214-3p mimic and inhibitor, transfected them into HepG2 cells and then administered drug stimulation. We found that, the role of PNS in reverting coagulation factors was further enhanced after transfection with miR-214-3p inhibitor compared to that in the inhibitor-NC group (Fig. 5D and E). Meanwhile, compared to the mimic-NC group, the effect of PNS on reversion of coagulation factors was reduced after transfection with the miR-214-3p mimic, and coagulation factor II and coagulation factor X showed the same change trend (Fig. 5F and G). Cellular proteins were extracted for Western-blot assay. The results showed that the protein levels of NR1I3 and CYP2C9 were down-regulated after transfection with the miR-214-3p mimic, while the elevated expression of NR1I3 and CYP2C9 caused by PNS was reverted (Fig. 5H). Meanwhile, after transfection with miR-214-3p inhibitor, the protein levels of NR1I3 and CYP2C9 were subsequently upregulated, while the elevated expression of NR1I3 and CYP2C9 due to PNS was more significant (Fig. 5I). These findings suggest that miR-214-3p is the key miRNA in the process of PNS affecting the pharmacodynamics of warfarin.

NR1I3 was localized in the cytoplasm in a phosphorylated state when inactive, and dephosphorylated and entered the nucleus when it was activated. To detect the activation effect of PNS on NR1I3, the cellular localization of NR1I3 was observed by immunofluorescence. The results showed that most of the control NR1I3 was located in the cytoplasm, while after PNS treatment, the entry of NR1I3 into the nucleus increased, suggesting that PNS has an activating effect on NR1I3 (Fig. 5J).

3.6. PNS modulation of miR-214-3p/NR1I3 resulting in drug interactions

To further verify the regulatory relationship between miR-214-3p and NR1I3 in the interaction between PNS and warfarin, we performed a reply experiment. HepG2 cells with knockdown NR1I3 were simultaneously transfected with miR-214-3p inhibitor, the changes of target genes NR1I3, CYP2C9 protein and mRNA levels were observed, and the changes of coagulation factors II and X were detected by Elisa. Western blotting and qRT-PCR results showed that the reduced expression of NR1I3 and CYP2C9 due to interference with NR1I3 was reverted after transfection with miR-214-3p inhibitor (Fig. 6A–D). Additionally, the inhibition of coagulation factors by warfarin was enhanced after interference with NR1I3, and this inhibition was partially restored after transfection with miR-214-3p inhibitor (Fig. 6E and F).

In addition, HepG2 cells overexpressing NR1I3 were simultaneously transfected with miR-214-3p mimic, and the results showed that the elevated expression of NR1I3, CYP2C9 caused by overexpression of NR1I3 was reverted by transfection with the miR-214-3p mimic (Fig. 6G–J). Meanwhile, the inhibitory effect of warfarin on coagulation factors was diminished after the overexpression of NR1I3, and this inhibitory effect was partially restored after transfection with miR-214-3p mimic (Fig. 6K and L). Therefore, together with the above results, we demonstrated that PNS modulation of miR-214-3p/NR1I3 affects the pharmacodynamics and pharmacokinetics of warfarin.

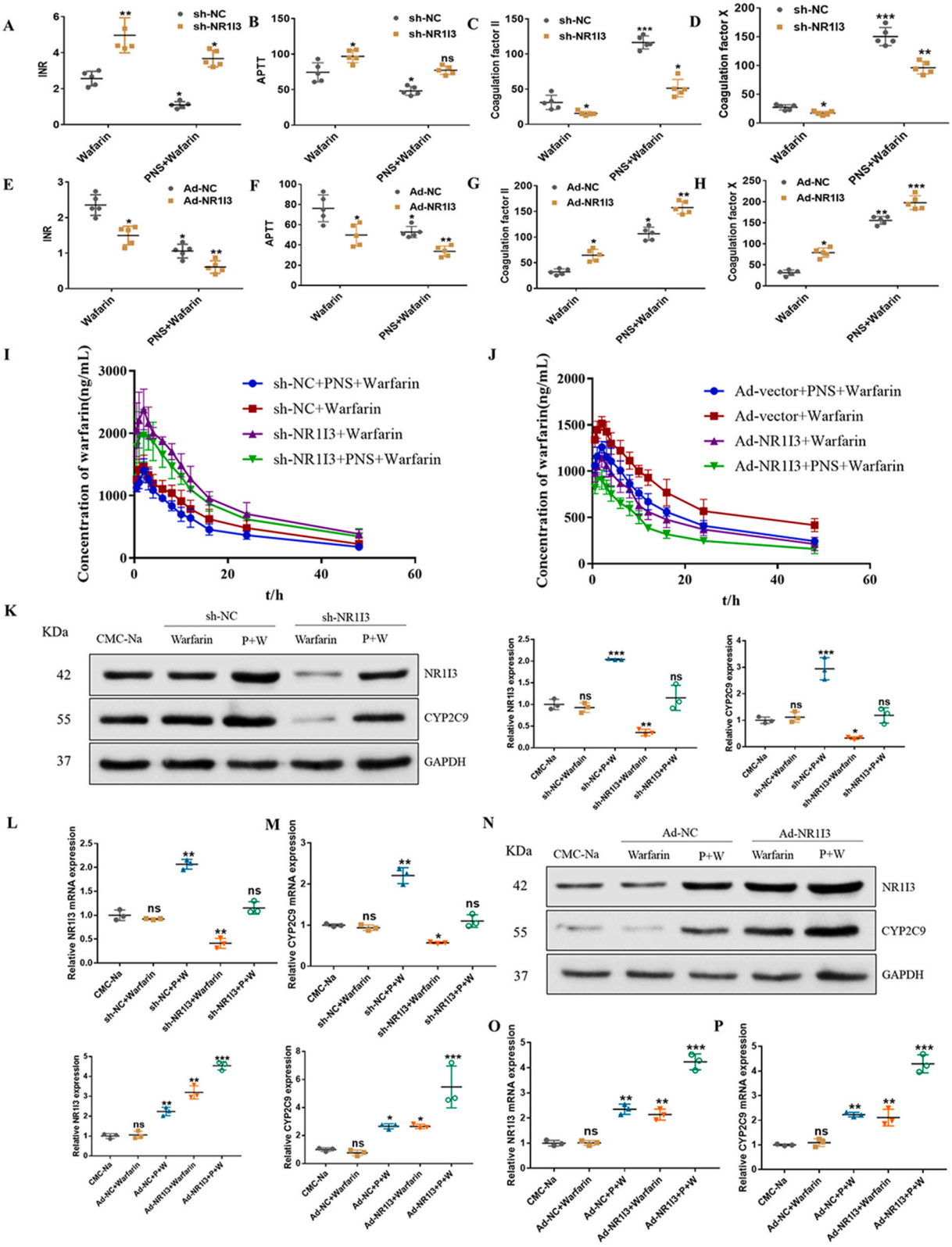

Fig. 4. (A–D: Changes in coagulation function and coagulation factors in rats treated by interfering with NR1I3 and feeding treatment, compared with the sh-NC + Warfarin group, n = 5; E–H: Changes in coagulation function and coagulation factors in rats treated by overexpressing NR1I3 and feeding treatment, compared with the Ad-NC + Warfarin group, n = 5; I: Changes in blood concentration and pharmacokinetics of warfarin in each group after interfering with NR1I3, the horizontal coordinate is the time from the last administration, and the vertical coordinate is the plasma drug concentration of warfarin in rats, n = 5; J: Changes in blood concentration and pharmacokinetics of warfarin in each group after overexpression of NR1I3, n = 5; K:Protein expression and statistics graphs of NR1I3 and CYP2C9 after interference NR1I3 and feeding treatment; L,M: Changes in NR1I3 and CYP2C9 mRNA expression after interference NR1I3 and dosing treatment; N: Protein expression and statistics graphs of NR1I3 and CYP2C9 after overexpression of NR1I3 and feeding treatment; O,P: Changes in NR1I3 and CYP2C9 mRNA expression after overexpression of NR1I3 and dosing treatment.)

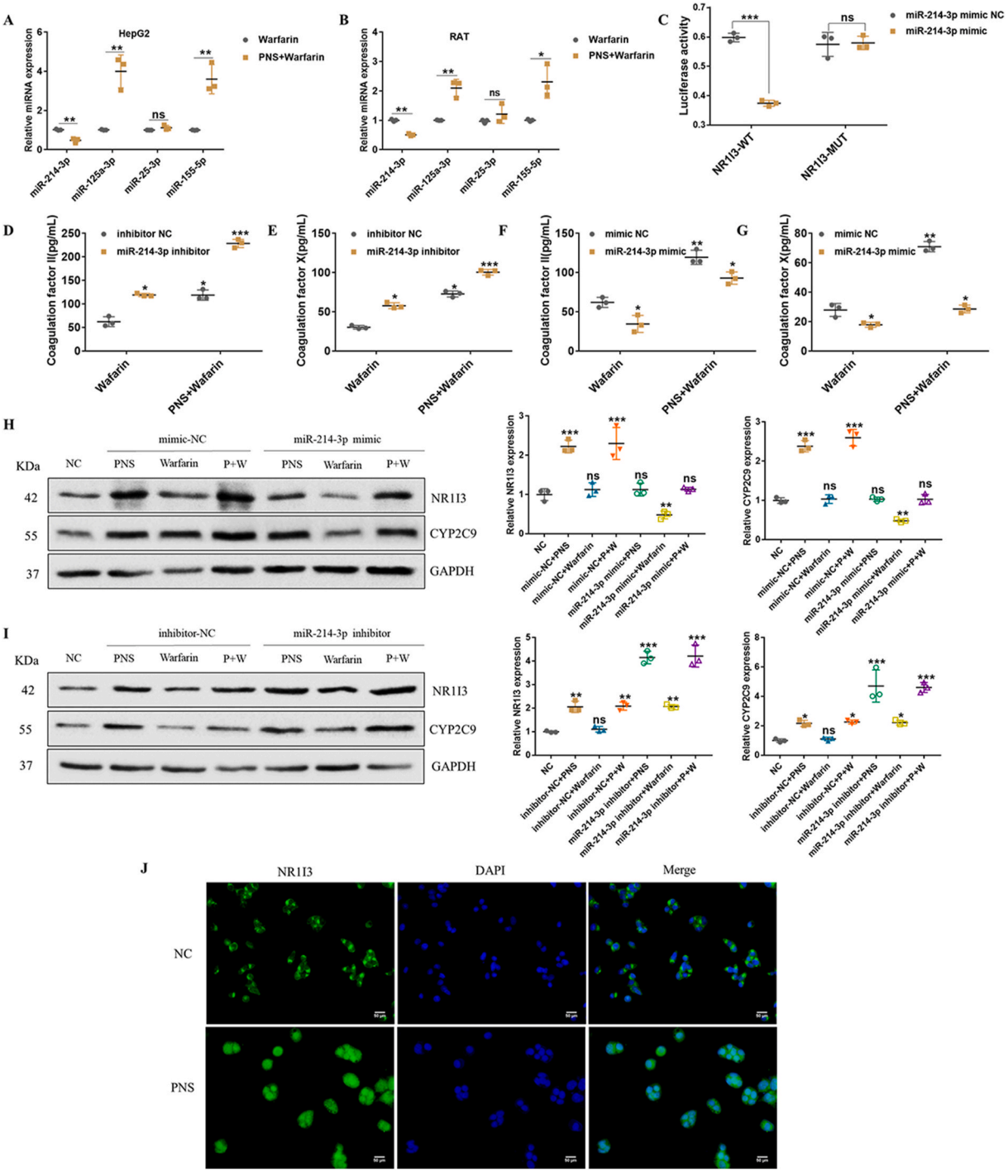

Fig. 5. (A: Differential expression of miRNAs in HepG2 cells after dosing treatment, n = 3; B: Differential expression of miRNAs in rat liver tissues after feeding treatment, n = 3; C: Luciferase reporter gene assay demonstrated direct binding of miR-214-3p and the 3′UTR of NR1I3 mRNA, n = 3; D,E: Changes in coagulation factor II and coagulation factor X detected by drug treatment after interfering with miR-214-3p, compared to the inhibitor NC + Warfarin group, n = 3; F,G: Overexpression of miR-214-3p followed by drug treatment to detect changes in coagulation factor II and coagulation factor X, compared to the mimic NC + Warfarin group, n = 3; H: Effect of overexpression of miR-214-3p on the protein expression levels of NR1I3, CYP2C9 and statistical graphs; I: Effect of interfering with miR-214-3p on the protein expression levels of NR1I3, CYP2C9 and statistical graphs; J: Cell fluorescence demonstrates that PNS activates NR1I3 into the nucleus, NR1I3 protein stains green, nucleus is stained with DAPI, scale bar: 50um.)

4. Discussion

Panax notoginseng, was expected to exhibit stronger anticoagulant effects when used in combination with warfarin, because it is described as the herb that "activates blood circulation and resolves blood stasis". However, in our clinical work we found that some patients with atrial fibrillation who were taking warfarin anticoagulation showed a significant downward trend in their INR values after concomitant oral administration of Panax ginseng, which elicited our interest. We first conducted animal experiments with PNS, the main active ingredient of Panax notoginseng, and warfarin as experimental drugs. The results of animal experiments were consistent with the phenomena observed in

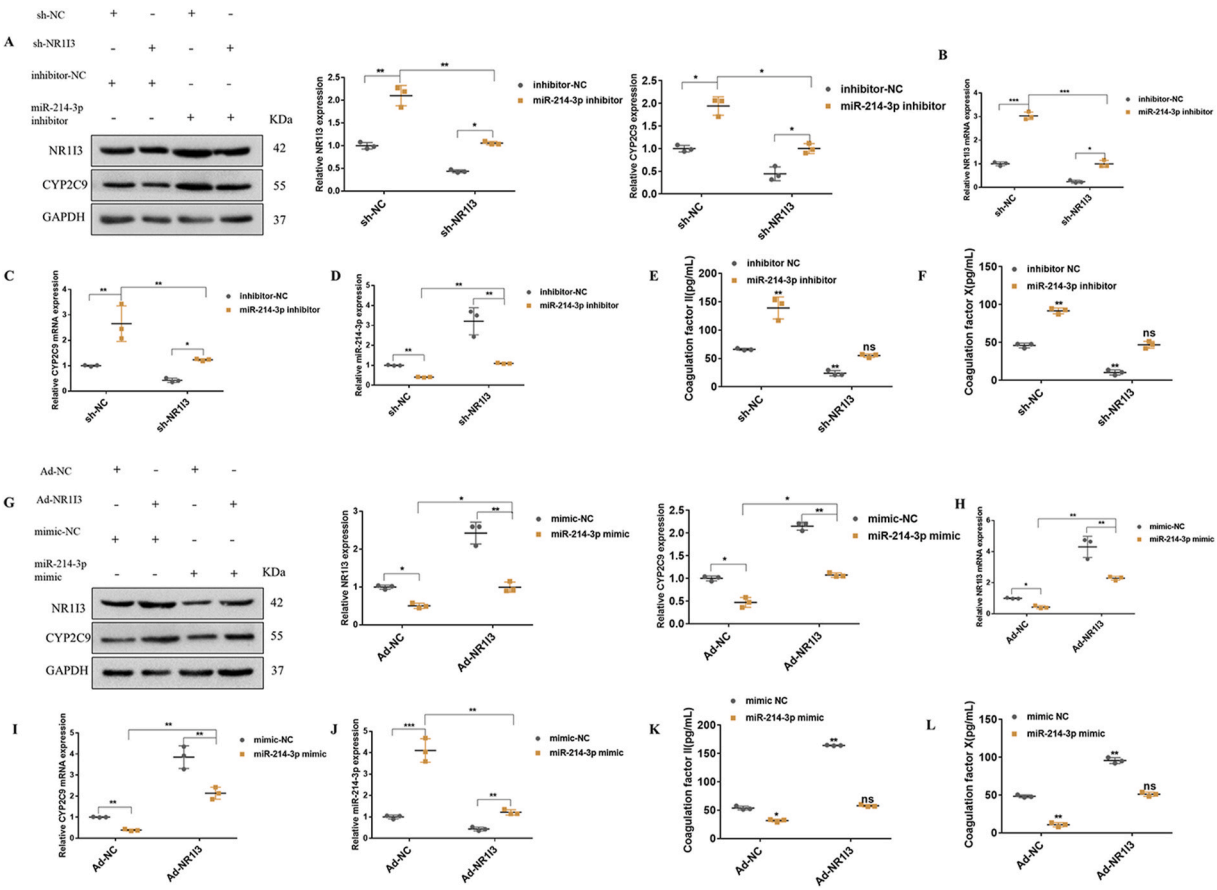

Fig. 6. (A: Transfection of miR-214-3p inhibitor while interfering with NR1I3, detection of changes in NR1I3, CYP2C9 protein levels and statistical graphs, n = 3; B–D: Transfection of miR-214-3p inhibitor while interfering with NR1I3, detection of changes in NR1I3, CYP2C9, miR-214-3p transcript levels, n = 3; E,F: Transfection of miR-214-3p inhibitor while interfering with NR1I3, and detect the changes of coagulation factors, compared with sh-NC + inhibitor NC group, n = 3; G: Transfection of miR-214-3p mimic while overexpressing NR1I3, detection of changes in NR1I3, CYP2C9 protein levels and statistical graphs, n = 3; H–J: Transfection of miR-214-3p mimic while overexpressing NR1I3, and detecting the changes in the transcript levels of NR1I3, CYP2C9, and miR-214-3p, n = 3; K,L: Transfection of miR-214-3p mimic while overexpressing NR1I3, and detect the changes of coagulation factors, compared with Ad-NC + mimic NC group, n = 3.)

our clinical work, in which PNS accelerated the metabolism and reduced the efficacy of warfarin in rats. To further investigate the mechanism of interaction between PNS and warfarin, we tested the main drug metabolizing enzymes of warfarin, CYP2C9, CYP1A2, CYP3A4 and the drug target VKORC1, and confirmed the induction of CYP2C9 by PNS.

Nuclear receptors are the largest known family of transcription factors that regulate gene expression. The constitutive androstane receptor (NR1I3, CAR) of the nuclear receptor family plays a key role in drug metabolism, energy homeostasis and cancer development by regulating the transcription of numerous target genes [5]. Although some studies have reported the induction of the drug metabolizing enzyme CYP450 by PNS, the specific mechanism of action has not been elucidated. Chen et al. demonstrated that NR1I3 as a transcription factor can activate the transcriptional regulation of CYP2C9 and upregulate its protein and mRNA expression [11]. The study by Assenat et al. demonstrated that in the liver, when NR1I3 expression was reduced, the transcriptional regulation of the drug metabolizing enzyme CYP2C9 was also reduced, further confirming that CYP2C9 is a target gene of NR1I3 [12]. Therefore, further studies are required to confirm whether PNS has a regulatory effect on NR1I3. Our results demonstrated that the expression of NR1I3 increased after PNS treatment, which was consistent with the trend of CYP2C9, suggesting that the effect of PNS on warfarin efficacy and pharmacokinetics was closely related to NR1I3. Our study demonstrated that PNS affects the efficacy and pharmacokinetics of warfarin through the induction of NR1I3.

With increasing research on non-coding RNAs, post-transcriptional modification of the nuclear receptor family by miRNAs has become one of the most mechanisms of their activation. MiR-21 and miR-27b were demonstrated by Kida et al. to affect the expression of important lipid metabolizing enzymes in the liver by post-transcriptionally regulating NR1C1 [13]. In breast cancer cell lines, miR-221/222 overexpression resulted in tumor cell resistance to tamoxifen (an estrogen receptor antagonist) treatment, whereas reduced levels of miR-221/222 and increased sensitivity of tumor cells to tamoxifen were associated with the mechanism of action of miRNAs that regulate estrogen receptors [14]. Thus, post-transcriptional regulation of nuclear receptors by miRNAs further affects the expression of the corresponding target genes of nuclear receptors. Our study demonstrated the direct binding effect of miR-214-3p and NR1I3, as well as the regulation of warfarin efficacy and pharmacokinetics by PNS through miR-214-3p/NR1I3.

Drug interactions are widespread in drug use, and stronger interactions can lead to adverse reactions, with the risk of drug interactions increasing as the number of combined drugs increases. Warfarin belongs to a class of anticoagulant drugs that are susceptible to food and other drugs, and the occurrence of interactions needs to be monitored more closely. In our study, we found that PNS accelerated the metabolism and reduced the efficacy of warfarin. We explored the mechanism of action of PNS in accelerating warfarin metabolism and reducing its efficacy at multiple levels, thereby providing a scientific basis for the management of clinical anticoagulation therapy with warfarin.

Funding

This work was partially supported by the Natural Science Foundation of Jiangxi Province (No.20232BAB216006) and Science and Technology Department of Jiangxi Province (No.20223BBG71010).

CRediT authorship contribution statement

Yuting Yang: Conceptualization, Formal analysis, Writing – original draft, Approval of the version of the manuscript to be published. **Zhenyu Zhai:** Formal analysis, Approval of the version of the manuscript to be published. **Huiming Yao:** Data curation, Approval of the version of the manuscript to be published. **Ling He:** Data curation, Approval of the version of the manuscript to be published. **Jun Shao:** Writing – original draft, Approval of the version of the manuscript to be published. **Zirong Xia:** Conceptualization, Approval of the version of the manuscript to be published. **Juxiang Li:** Conceptualization, revising the manuscript critically for important intellectual content, Approval of the version of the manuscript to be published.

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgements

All persons who have made substantial contributions to the work reported in the manuscript (e.g., technical help, writing and editing assistance, general support), but who do not meet the criteria for authorship, are named in the Acknowledgements and have given us their written permission to be named. If we have not included an Acknowledgements, then that indicates that we have not received substantial contributions from non-authors.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2024.05.003.

References

[1] Whitlon D, Sadowski J, Suttie J. Mechanism of coumarin action: significance of vitamin K epoxide reductase inhibition. Biochemistry 1978;17(8):1371–7.
[2] Popov Aleksandrov A, Mirkov I, Ninkov M, et al. Effects of warfarin on biological processes other than haemostasis: a review. Food Chem Toxicol: an international journal published for the British Industrial Biological Research Association 2018; 113:19–32.
[3] Dong T, Cui X, Song Z, et al. Chemical assessment of roots of Panax notoginseng in China: regional and seasonal variations in its active constituents. J Agric Food Chem 2003;51(16):4617–23.
[4] Liu Y, Liu T, Zhao J, et al. Phospholipase Cγ2 signalling contributes to the haemostatic effect of Notoginsenoside Ft1. J Pharm Pharmacol 2019;71(5):878–86.
[5] Qatanani M, Moore D. CAR, the continuously advancing receptor, in drug metabolism and disease. Curr Drug Metabol 2005;6(4):329–39.
[6] Mutoh S, Osabe M, Inoue K, et al. Dephosphorylation of threonine 38 is required for nuclear translocation and activation of human xenobiotic receptor CAR (NR1I3). J Biol Chem 2009;284(50):34785–92.
[7] Gardner-Stephen D, Heydel J, Goyal A, et al. Human PXR variants and their differential effects on the regulation of human UDP-glucuronosyltransferase gene expression. Drug metabolism and disposition: the biological fate of chemicals 2004; 32(3):340–7.
[8] Deng Y, Wei Z, Huang M, et al. Long non-coding RNA F11-AS1 inhibits HBV-related hepatocellular carcinoma progression by regulating NR1I3 via binding to microRNA-211-5p. J Cell Mol Med 2020;24(2):1848–65.
[9] Wang Y, Yan L, Liu J, et al. αThe HNF1-regulated LncRNA HNF1-AS1 is involved in the regulation of cytochrome P450 expression in human liver tissues and Huh7 cells. J Pharmacol Exp Therapeut 2019;368(3):353–62.
[10] Illanes S, Zhou W, Heiland S, Markus Z, Veltkamp R. Kinetics of hematoma expansion in murine warfarin-associated intracerebral hemorrhage. Brain Res 2010;1320:135–42.
[11] Chen Y, Kissling G, Negishi M, Goldstein JA. The nuclear receptors constitutive androstane receptor and pregnane X receptor cross-talk with hepatic nuclear factor 4alpha to synergistically activate the human CYP2C9 promoter. J Pharmacol Exp Ther 2005;314(3):1125–33.
[12] Assenat E, Gerbal-Chaloin S, Larrey D, et al. Interleukin 1beta inhibits CAR-induced expression of hepatic genes involved in drug and bilirubin clearance. Hepatology 2004;40(4):951–60.
[13] Kida K, Nakajima M, Mohri T, et al. PPARα is regulated by miR-21 and miR-27b in human liver. Pharm Res (N Y) 2011;28(10):2467–76.
[14] Zhao JJ, Lin J, Yang H, et al. MicroRNA-221/222 negatively regulates estrogen receptor alpha and is associated with tamoxifen resistance in breast cancer. J Biol Chem 2008;283(45):31079–86.