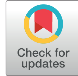

Available online at www.sciencedirect.com

ScienceDirect

Journal of the Chinese Medical Association 81 (2018) 324−330

www.jcma-online.com

Original Article

Warfarin accelerated vascular calcification and worsened cardiac dysfunction in remnant kidney mice

Ming-Tsun Tsai [a,b], Ying-Ying Chen [b,c], Wei-Jeng Chang [d], Szu-Yuan Li [b,c,*]

[a] Division of Nephrology, Department of Medicine, Taipei Veterans General Hospital Taoyuan Branch, Taoyuan, Taiwan, ROC
[b] National Yang-Ming University, School of Medicine, Taipei, Taiwan, ROC
[c] Division of Nephrology, Department of Medicine, Taipei Veterans General Hospital, Taipei, Taiwan, ROC
[d] National Laboratory Animal Center, National Applied Research Laboratories, Taipei, Taiwan, ROC

Received June 23, 2017; accepted August 7, 2017

Abstract

Background: Vascular calcification is highly prevalent in end-stage renal disease (ESRD) and is a significant risk factor for future cardiovascular events and death. Warfarin use results in dysfunction of matrix Gla protein, an inhibitor of vascular calcification. However, the effect of warfarin on vascular calcification in patients with ESRD is still not well characterized. Thus we investigated whether arterial calcification can be accelerated by warfarin treatment both *in vitro* and *in vivo* using a mouse remnant kidney model.
Methods: Human aortic smooth muscle cells (HASMC) were cultured in medium supplemented with warfarin and phosphate to investigate the potential role of this drug in osteoblast transdifferentiation. For *in vivo* study, adult male C57BL/6 mice underwent 5/6 nephrectomy were treated with active vitamin D3 plus warfarin to determine the extent of vascular calcification and parameters of cardiovascular function.
Results: We found that the expressions of Runx2 and osteocalcin in HASMC were markedly enhanced in the culture medium containing warfarin and high phosphate concentration. Warfarin induced calcification of cultured HASMC in the presence of high phosphate levels, and this effect is inhibited by vitamin K2. Severe aortic calcification and reduced left ventricular ejection fractions were also noted in 5/6 nephrectomy mice treated with warfarin and active vitamin D3.
Conclusion: Warfarin treatment contributes to the accelerated vascular calcification in animal models of advanced chronic kidney disease. Clinicians should therefore be aware of the profound risk of warfarin use on vascular calcification and cardiac dysfunction in patients with ESRD and atrial fibrillation.
Copyright © 2018, the Chinese Medical Association. Published by Elsevier Taiwan LLC. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Keywords: Left ventricular dysfunction; Uremia; Vascular calcification; Warfarin

1. Introduction

Atrial fibrillation (Af) is the most common cardiac dysrhythmia and is associated with a significant increased risk of stroke as well as subsequent cardiovascular (CV) morbidity and mortality.[1,2] The prevalence of Af increases with age, reaching approximately 8% in those aged ≥80 years compared with.4%−1.0% in the general population.[3] In patients with end-stage renal disease (ESRD), Af is common in the post-dialysis period, with a prevalence ranging from about 10% to 20% depending on both the definition and age of the population studied.[4] For the general population, anticoagulation therapy can substantially reduce the risk of stroke in patients with Af and a CHA2DS2-VASc score of more than two.[5,6] However, there are few prospective clinical trials about the protective effect of warfarin in patients with ESRD, and the

Conflicts of interest: The authors declare that they have no conflicts of interest related to the subject matter or materials discussed in this article.

* Corresponding author. Dr. Szu-Yuan Li, Division of Nephrology, Department of Medicine, Taipei Veterans General Hospital, 201, Section 2, Shi-Pai Road, Taipei 112, Taiwan, ROC.
E-mail address: syli@vghtpe.gov.tw (S.-Y. Li).

https://doi.org/10.1016/j.jcma.2017.08.021
1726-4901/Copyright © 2018, the Chinese Medical Association. Published by Elsevier Taiwan LLC. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

benefit of warfarin for stroke prevention in hemodialysis (HD) patients with Af is still debated in most retrospective studies.[7–9] Warfarin may cause excessive bleeding and calcification in vascular structures that can be life-threatening and even lead to death in patients with advanced chronic kidney disease (CKD).[7] Accordingly, the Kidney Disease Improving Global Outcomes (KDIGO) guideline suggests that routine anticoagulation in ESRD patients with Af for the primary prevention of stroke is not indicated because of these complications and lack of valid evidence for stroke prevention.[10]

In addition, vascular calcification is highly prevalent in ESRD and is a significant risk factor for future CV events and death. Matrix Gla protein (MGP) is a vitamin K–dependent protein highly expressed in bone and arteries, where it acts as a local regulator of vascular calcification.[11] Warfarin use results in undercarboxylation of MGP and impairs its biological effects.[12] This effect on MGP may explain the more rapid progression of coronary calcification in patients taking warfarin.[13] However, the effect of warfarin on vascular calcification and CV outcomes in patients with ESRD is still not well characterized. Thus, we investigated whether arterial calcification can be accelerated by warfarin treatment both *in vitro* and *in vivo* using a mouse remnant kidney model.

2. Methods

2.1. Ethics statement

The experimental animal procedures were conducted in accordance with the *European Commission Directive 86/609/EEC for animal experiments* and were approved by the Institutional Animal Care and Use Committee of the study hospital.

2.2. Cell culture and induction of calcification

Human aortic vascular smooth muscle cells (HASMC) were obtained from Cambrex Bioscience (Wokingham, United Kingdom) and cultured in medium-199 (Sigma–Aldrich, St. Louis, MO) containing 15% fetal bovine serum (FBS), 2 mmol/L L-glutamine, 100 U/mL penicillin G, and 100 μg/mL streptomycin. Calcification of HASMC cultures was induced by the method of Wada et al.[14] Briefly, HASMC were cultured in normal growth medium for 4 days and then switched to medium containing vehicle control, high phosphate levels (final concentration 5 mM), warfarin (10 μM; Sigma–Aldrich, St. Louis, MO), or warfarin plus high phosphate levels for 10 days. The cell culture medium was replaced with fresh medium every other day. The antagonistic effect of vitamin K1 (phytomenadione, 5 μM; Sigma–Aldrich, St. Louis, MO) and vitamin K2 (menaquinone, 25 μM; Sigma–Aldrich, St. Louis, MO) on mineral deposition in HASMC induced by warfarin was also examined. The extent of transdifferentiation from HASMC into osteoblasts was evaluated using α-smooth muscle actin, osteocalcin, and Runx2 levels that were examined by immunoblotting as described below. Cell cultures were stained for mineral deposition using the von Kossa method as previously described.[14]

2.3. Western blot to detect osteocalcin and Runx2

Expression levels of both osteocalcin and Runx2 were evaluated using cell lysate that was electrophoresed in 12.5% SDS-PAGE. Western blotting was performed with a rabbit polyclonal anti-human α-smooth muscle actin antibody at a 1:400 dilution (Abcam, Cambridge, UK), rabbit polyclonal anti-human osteocalcin antibody at a 1:2000 dilution (Abcam, Cambridge, UK) or with rabbit anti-human Runx2 antibody (Invitrogen, Carlsbad, CA) at a 1:500 dilution followed by horseradish peroxidase–labeled anti-rabbit IgG antibody. Antigen-antibody complexes were visualized with the horseradish peroxidase chemiluminescence system (GE Healthcare, Buckinghamshire, UK).

2.4. Animals

Twelve-week-old male C57BL/6 mice were purchased from the National Laboratory Animal Center (Taipei, Taiwan) and allowed free access to standard rodent chow and water. Animals were housed in a temperature-controlled and light-controlled environment (23 °C, 50 ± 5% humidity and a 12-h light/dark cycle).

2.5. 5/6 Nephrectomy and study groups

The remnant kidney model was induced in animals following the two-step surgical procedure under Avertin anesthesia. Briefly, the left kidney was exposed, and the upper and lower poles were electrocauterized. Two weeks later, a right total nephrectomy was performed.

The C57BL/6 mouse strain is known to be resistant to the development of vascular calcification, despite uremic status induced by both the 5/6 nephrectomy and feeding with high dietary phosphate.[15] In experimental animal models, excessive dietary intake of active vitamin D3 may induce vascular calcification via complex, systemic feedback regulatory mechanisms that control calcium-phosphate metabolism.[16,17] Therefore, we evaluated the effects of warfarin on both arterial calcification and cardiovascular function in mice that underwent a 5/6 nephrectomy and were fed a diet containing high-dose active vitamin D3.

At 3 days post-5/6 nephrectomy, mice were divided into five groups of 10–12 mice per group: the sham-operated control group fed vehicle, the 5/6 nephrectomy group fed vehicle, the 5/6 nephrectomy group fed warfarin (3 mg/kg/day), the 5/6 nephrectomy group fed active vitamin D3 (1α,25-dihydroxyvitamin D3,.1 μg/kg/day; Sigma–Aldrich, St. Louis, MO), and the 5/6 nephrectomy group fed warfarin plus active D3. All mice were orally administered vitamin K1 (Sigma–Aldrich, St. Louis, MO) to prevent internal bleeding and were sacrificed 16 weeks after the 5/6 nephrectomy.

2.6. Serum chemistries

Blood collection by cardiac puncture was performed at the time of sacrifice. Serum levels of blood urea nitrogen (BUN), creatinine, phosphate, and calcium were analyzed by standard autoanalyzer methods.

2.7. Aortic calcification detection in micro computed tomography (micro-CT)

All groups of mice underwent micro-CT analysis on a monthly basis throughout the study. Ascending and descending aortas were scanned using a 1076 SkyScan micro-CT scanner (SkyScan, Kontich, Belgium) and both reconstructed and analyzed with CTAn and NRecon software. The imaging protocol consisted of respiratory and cardiac gating operated at 80 kV and 32 mA. Images were acquired at a resolution of 9 μm with a rotation step of.25°.

2.8. M-mode echocardiography

All mice underwent transthoracic echocardiography at baseline and before sacrifice. Echocardiography was performed with a Flex Focus 1202 imaging system (BK Ultrasound, MA, USA). We measured the left ventricular (LV) anterior wall diastolic thickness, LV posterior wall diastolic thickness, LV end-diastolic diameter, and LV end-systolic diameter at the M-mode, as previously described.[18] The LV ejection fraction (LVEF) was then calculated from measurements of chamber diameters and wall thickness.

2.9. Quantitative analysis of aortic calcium

At termination, mice were sacrificed, and aortas were collected for quantitation of calcium deposition and histological analysis. Dissected descending aortic tissues were frozen, lyophilized, and decalcified with 500-fold excess of 10% formic acid for 24 h. The calcium content of the supernatant was determined with the colorimetric o-cresolphthalein method (Randox Laboratories Ltd., Crumlin, UK) as per the manufacturer's instructions. Tissue calcium content was expressed as microgram calcium/gram dry weight.

2.10. Histological analysis of aortic vessels

For histological analysis, 4% paraformaldehyde-fixed tissue specimens were embedded in paraffin. Sections were cut at a thickness of 5 μm, and the von Kossa staining method was used to quantify calcium deposition. Stained areas were measured using an ImageJ computer program (National Institutes of Health, USA).

2.11. Statistical analysis

Data are presented as means ± SEM. Comparisons among groups were performed by an ANOVA analysis followed by a Bonferroni's post hoc test. All p values were two-sided, and a p value of.05 or less was considered to be statistically significant.

3. Results

3.1. Augmentation of phosphate-induced osteogenic transformation of HASMC by warfarin

To develop an *in vitro* experimental model, HASMC were cultured in high-phosphate medium. Warfarin significantly increased the frequency of vascular calcification in patients with ESRD; therefore, we asked whether warfarin promoted the phenotype transformation of HASMC into osteoblast/osteocyte-like cells. Treatment of HASMC grown in normal growth medium with warfarin did not result in increased expression of Runx2 (a key transcription factor associated with osteoblast differentiation) and osteocalcin (a protein secreted solely by osteoblasts) as shown by Western blot (Fig. 1A). However, treatment with warfarin resulted in augmentation of phosphate-induced osteogenic transdifferentiation of HASMC, as demonstrated by the upregulation of both osteocalcin and Runx2 as well as by a substantial decrease in the expression of α-smooth muscle actin (Fig. 1A).

Von Kossa staining was performed on Day 10 to confirm the occurrence of mineral deposition in these cultures (Fig. 1B). Calcium deposits developed in HASMC grown in high-phosphate medium but not in the control culture grown in normal cell culture medium. We found that the addition of warfarin alone to the normal growth medium did not promote calcium deposition. However, warfarin can significantly increase calcium deposits in HASMC grown in high-phosphate medium. We found it interesting that the addition of vitamin K2 but not vitamin K1 to the high-phosphate medium was able to inhibit the influence of warfarin-augmented calcium deposition in HASMC (Fig. 1B).

3.2. Aortic calcification was potentiated by cotreatment with warfarin and active vitamin D3 in a remnant kidney mouse model

Serum biochemical parameters in all mice are presented in Table 1. Serum concentrations of calcium and phosphate were significantly increased in mice that underwent 5/6 nephrectomy and were treated with daily active vitamin D3 compared with those that underwent 5/6 nephrectomy alone ($p < 0.05$). The administration of warfarin did not affect serum levels of these ions in 5/6 nephrectomy mice treated with active vitamin D3.

We scanned all animals with micro-CT to evaluate the extent of aortic calcification on a monthly basis. Multiple small spots of calcification were detected two months after surgery in the aorta from the 5/6 nephrectomy mice simultaneously treated with warfarin and active vitamin D3. Extensive vascular calcifications in the ascending and descending aorta on micro-CT scans were recognized only in remnant kidney mice administered warfarin and active vitamin D3 at the end of the fourth month (Fig. 2A and B).

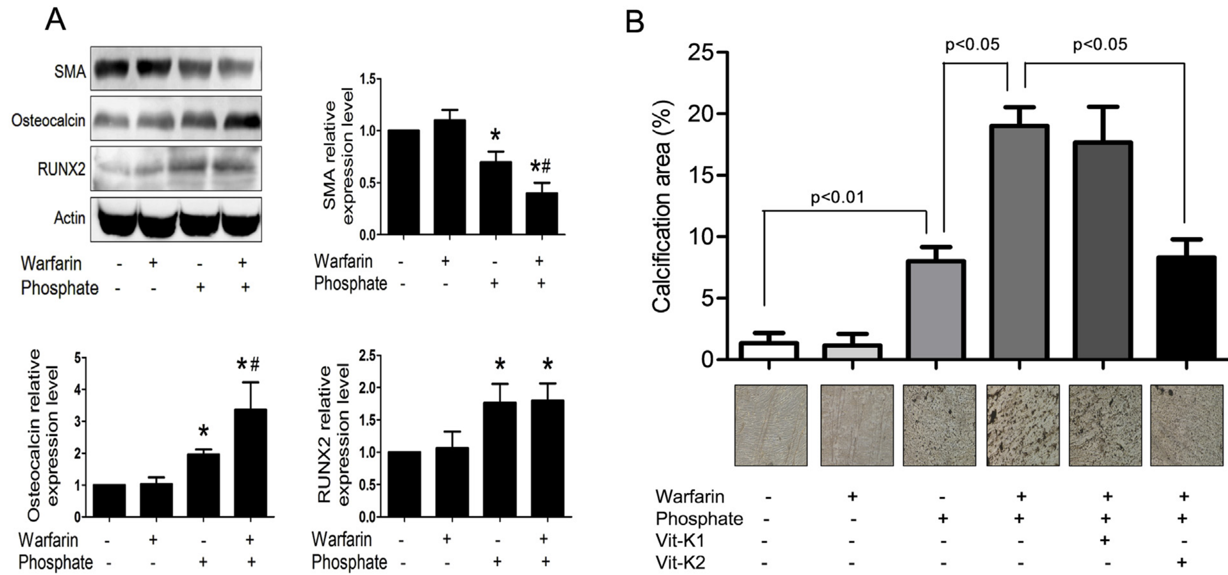

Fig. 1. **Warfarin augments phosphate-induced osteogenic transformation of HASMC.** HASMC were cultured in normal growth medium or in high-phosphate medium in the absence or presence of warfarin (10 μM) for 10 days. (A) Representative Western blots, and quantitative analysis of Western blots. The expression of α-smooth muscle actin, osteocalcin and Runx2 were measured as described in the Method section. Data are means ± SEM of three independent experiments each performed in duplicate. *$p < 0.05$ compared to the cells grown in normal medium. #$p < 0.05$ compared to the cells grown in high-phosphate medium. (B) Von Kossa staining shows diffusive calcification predominantly in HASMC cultured in the presence of high-phosphate medium (×100 magnification). The area of calcification is further increased in the presence of warfarin. This augmentation of warfarin for calcification was abolished by vitamin K2. Data are means ± SEM of three independent experiments each performed in duplicate. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Comparison of serum biochemistry among different groups of mice.

Group	BUN (mg/dL)	Creatinine (mg/dL)	Calcium (mg/dL)	Phosphate (mg/dL)
Sham operation (n = 10)	25.1 ± 3.5	0.12 ± 0.04	10.6 ± 0.6	8.9 ± 1.3
5/6 Nx (n = 10)	82.1 ± 6.5**	1.22 ± 0.47**	10.7 ± 0.2	9.0 ± 1.5
5/6 Nx + Warfarin (n = 11)	85 ± 8.2**	1.33 ± 0.28**	11.9 ± 0.9	9.1 ± 1.2
5/6 Nx + Vit-D_3 (n = 12)	84.2 ± 7.5**	1.44 ± 0.35**	15.2 ± 0.4*#	12.6 ± 1.5*#
5/6 Nx + Vit-D_3 + Warfarin (n = 11)	86.5 ± 8.5**	1.42 ± 0.37**	15.7 ± 0.8*#	12.8 ± 1.7*#

Abbreviations: 5/6 Nx = 5/6 nephrectomy; BUN = blood urine nitrogen.
**$p < 0.01$ compared to the sham operation group.
*$p < 0.05$ compared to the sham operation group.
#$p < 0.05$ compared to the 5/6 Nx group.

Both histologic findings and biochemical analyses of aortic calcium were consistent with imaging findings described above (Fig. 2C and D). Aorta from 5/6 nephrectomy mice simultaneously treated with these two agents demonstrated significant medial calcification as detected by von Kossa staining.

3.3. Warfarin treatment may further compromise cardiac function in 5/6 nephrectomy mice that were administered with active vitamin D3

Transthoracic echocardiography was performed on all mice prior to sacrifice. Compared with sham-operated mice, LVEF in 5/6 nephrectomy mice decreased significantly ($p < 0.05$, Fig. 3A and B). There was no significant difference in cardiac function between 5/6 nephrectomy mice treated with warfarin and those that underwent nephrectomy alone.

However, warfarin treatment may further compromise LVEF in 5/6 nephrectomy mice that were administered active vitamin D3 compared with 5/6 nephrectomy controls ($p < 0.05$, Fig. 3A and B).

4. Discussion

In this study, we demonstrated that warfarin treatment might potentiate the transformation of HASMC into osteoblast-like cells in the presence of excess phosphorus. Warfarin can cause dramatic mineral deposition in cells grown in high-phosphate medium. In addition, in the hyperphosphatemic environment induced by the use of active vitamin D3, progression of aortic calcification was greater in 5/6 nephrectomy mice administered warfarin. Cardiac dysfunction was also both more common and severe in this group of mice.

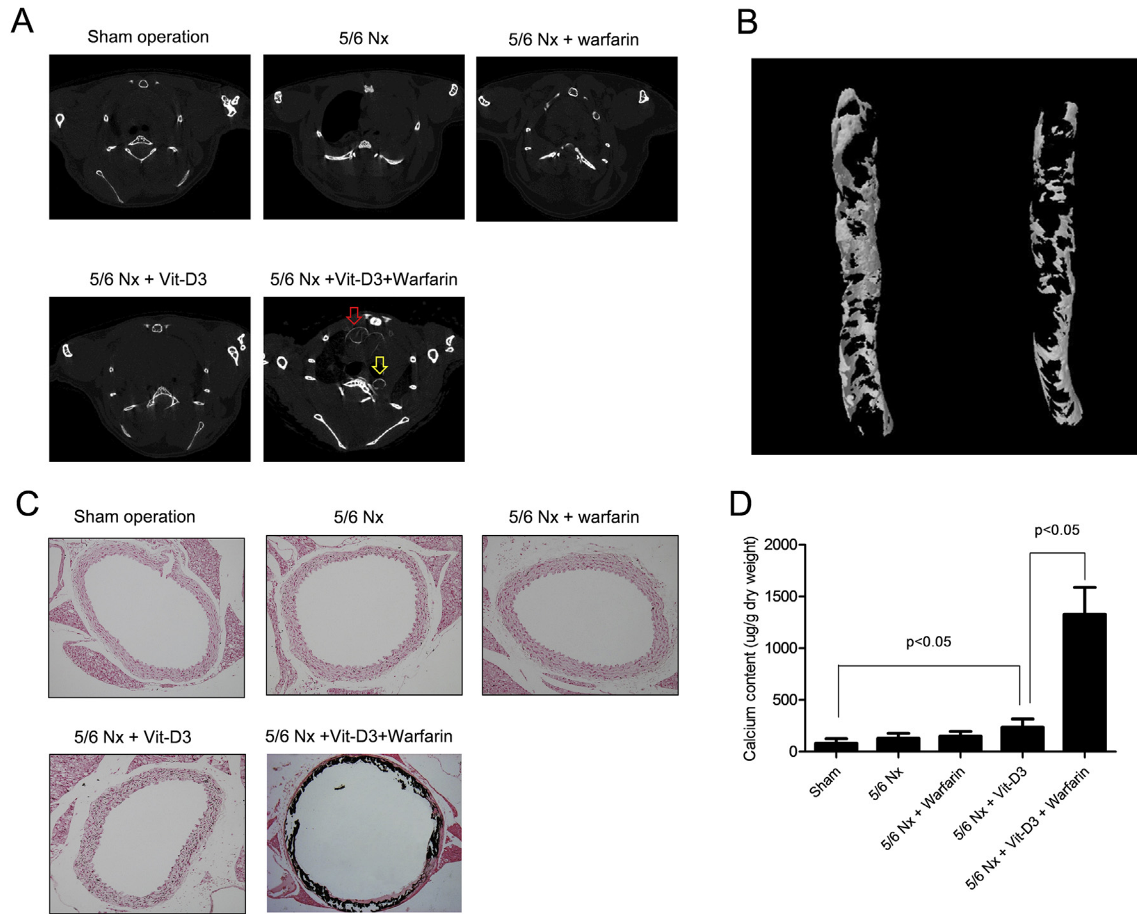

Fig. 2. **Aortic calcification was potentiated by cotreatment with warfarin and active vitamin D3 in a remnant kidney mouse model.** (A) Micro-CT of the chest showing extensive calcification of ascending and descending aorta in 5/6 nephrectomy mice simultaneously treated with warfarin and active vitamin D3. The red arrow indicates ascending aorta, and the yellow arrow indicates descending aorta. (B) Three-dimensional image reconstruction technologies can reveal extensive calcification in the aortic wall, which had become like a "lead pipe" from mice underwent 5/6 nephrectomy that are treated with warfarin and active vitamin D3. (C) All mice were sacrificed for tissue harvest after four months. The aorta was extracted, and the serial sections were subjected to pathological analysis. Medial calcification was detected by von Kossa staining in 5/6 nephrectomy mice treated with warfarin and active vitamin D3. (D) Aortic calcium content in 5/6 nephrectomy mice and sham controls under treatment with or without warfarin, active vitamin D3 or warfarin plus active vitamin D3. Bars represent mean ± SEM (10–12 mice per group).

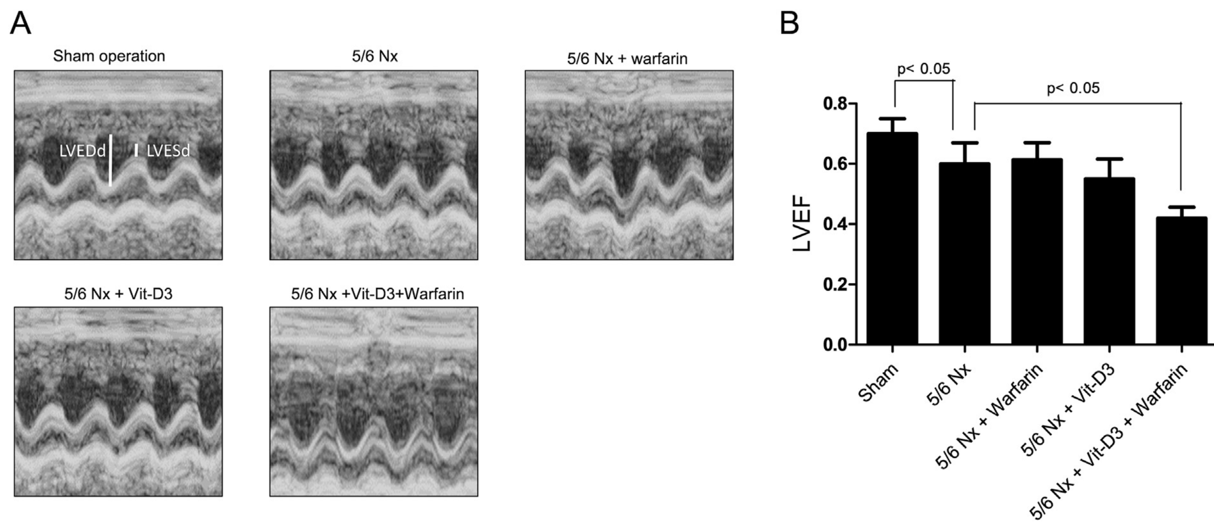

Fig. 3. **Warfarin treatment may further compromise cardiac function in 5/6 nephrectomy mice that were administered with active vitamin D3.** (A) Representative M-mode echocardiographic recordings. (B) Quantitative assessment of LVEF. Warfarin treatment may further compromise LVEF in 5/6 nephrectomy mice that were administered with active vitamin D3 compared to 5/6 nephrectomy controls ($p < 0.05$). Bars represent mean ± SEM. Abbreviations: LVESd: left ventricular end-systolic diameter; LVEDd: left ventricular end-diastolic diameter.

The optimal management of Af in patients with ESRD is controversial as some investigators have raised concerns about the potential risk of cardiovascular calcification with warfarin. Over the past decades, several studies have demonstrated that vascular calcification is highly prevalent in ESRD and is associated with an increased risk of both CV disease and death.[19] Arterial calcification can be broadly classified into two categories: intimal atherosclerotic calcification and medial arterial calcification. Intimal calcification is associated with fibro-fatty type atherosclerotic plaque formation, which can protrude into the vascular lumen and subsequently lead to ischemic heart disease.[20] Medial calcification is a form of arteriosclerosis, where circumferential distribution of calcium deposits is found in the tunica media of elastic arteries. This form of calcification is commonly found in patients with ESRD.[21] Medial calcification can increase arterial stiffness and is linked to the risk of subsequently developing heart failure, valvular sclerosis, and ventricular hypertrophy in ESRD patients treated with dialysis.[22,23] An improved understanding of the mechanisms underlying vascular calcification is needed to design appropriate therapeutic strategies for these patients.

As an important transcription factor, Runx2 is necessary for normal bone development. This factor also plays a critical role in the pathophysiology of vascular calcification.[24] Blocking the activities of Runx2 significantly decreased uremic serum-induced expression of both alkaline phosphatase and osteocalcin in vascular smooth muscle cells (VSMC) *in vitro*.[25] Elevated phosphate may upregulate Runx2 expression in VSMC thereby promoting the fate of transdifferentiation of these cells into osteoblast-like cells, with subsequent calcification *in vitro*.[24]

Similarly, there are many calcification inhibitors that regulate each step of extraskeletal calcification in patients undergoing dialysis.[26] Among these factors, MGP has received considerable attention. This calcification inhibitor may be linked to the observed increase in warfarin-induced artery calcification in patients with ESRD.[26,27] There are two forms of vitamin K: vitamin K1 and vitamin K2. Vitamin K1 is usually processed in the liver and facilitates the activation of the coagulation cascade, whereas Vitamin K2 has a systemic distribution, having a specific role in osteoblast and vascular smooth muscle cell function.[28] Both K1 and K2 are inhibited by warfarin administration.[27] Vitamin K2 carboxylates MGP in the vasculature where MGP acts as an inhibitor of vascular calcification by binding calcium ions and subsequently inhibiting mineral deposition.[11] Nutritional vitamin K deficiency and warfarin use, therefore, lead to impaired carboxylation of MGP.[12] Our *in vitro* and *in vivo* results are consistent with these findings.

Our results demonstrated that warfarin did not directly induce osteogenic transformation of HASMC and caused aortic calcification in mice with 5/6 nephrectomy under normal phosphate conditions. On the other hand, aortic calcification and cardiac dysfunction were potentiated by treatment with warfarin in 5/6 nephrectomy mice that exhibited both hypercalcemia and hyperphosphatemia. This result is consistent with the findings of several previous clinical studies of anticoagulation therapy in patients with CKD.[29,30] A recent meta-analysis has offered evidence that warfarin therapy for Af may have an unfavorable risk/benefit ratio in patients with ESRD but not in those with non-end-stage CKD.[30] Furthermore, Nigwekar et al. demonstrated that higher serum levels of calcium and phosphorus, nutritional vitamin D use, and warfarin treatments at the initiation of hemodialysis were associated with increased odds of developing future calcific uremic arteriolopathy.[31] Our previous study[32] found that the total mortality rate of ESRD-Af patients was not associated with the use of warfarin. In contrast, in hemodialysis patients, warfarin use was associated with an increased incidence of congestive heart failure and peripheral artery disease. We suggest that warfarin should be used with caution in patients with ESRD, especially in those with poor control of CKD−mineral bone disorder.

In conclusion, the results of our study suggest that warfarin treatment contributes to accelerated vascular calcification in mice with advanced chronic kidney disease treated with calcitriol. Therefore, clinicians should be aware of the profound risk of warfarin use on vascular calcification and cardiac dysfunction in patients with both ESRD and Af.

Acknowledgments

This study was supported in part by research grants from Taipei Veterans General Hospital (V105C-013).

References

1. Kannel WB, Abbott RD, Savage DD, McNamara PM. Epidemiologic features of chronic atrial fibrillation: the Framingham study. *N Engl J Med* 1982;**306**:1018−22.
2. Feinberg WM, Blackshear JL, Laupacis A, Kronmal R, Hart RG. Prevalence, age distribution, and gender of patients with atrial fibrillation. Analysis and implications. *Arch Intern Med* 1995;**155**:469−73.
3. Fuster V, Rydén LE, Cannom DS, Crijns HJ, Curtis AB, Ellenbogen KA, et al. ACC/AHA/ESC 2006 guidelines for the management of patients with atrial fibrillation-executive summary: a report of the American college of cardiology/American heart association task force on practice guidelines and the European society of cardiology committee for practice guidelines (writing committee to revise the 2001 guidelines for the management of patients with atrial fibrillation). *Eur Heart J* 2006;**27**: 1979−2030.
4. Winkelmayer WC, Patrick AR, Liu J, Brookhart MA, Setoguchi S. The increasing prevalence of atrial fibrillation among hemodialysis patients. *J Am Soc Nephrol* 2011;**22**:349−57.
5. Estes 3rd NA, Halperin JL, Calkins H, Ezekowitz MD, Gitman P, Go AS, et al. ACC/AHA/physician consortium 2008 clinical performance measures for adults with nonvalvular atrial fibrillation or atrial flutter: a report of the American college of cardiology/American heart association task force on performance measures and the physician consortium for performance improvement (writing committee to develop clinical performance measures for atrial fibrillation) developed in collaboration with the heart rhythm society. *J Am Coll Cardiol* 2008;**51**:865−84.
6. Anderson JL, Halperin JL, Albert NM, Bozkurt B, Brindis RG, Curtis LH, et al. Management of patients with atrial fibrillation (compilation of 2006 ACCF/AHA/ESC and 2011 ACCF/AHA/HRS recommendations): a report of the American college of cardiology/American heart association task force on practice guidelines. *J Am Coll Cardiol* 2013;**61**:1935−44.

7. Olesen JB, Lip GY, Kamper AL, Hommel K, Køber L, Lane DA, et al. Stroke and bleeding in atrial fibrillation with chronic kidney disease. *N Engl J Med* 2012;**367**:625–35.
8. Chan KE, Lazarus JM, Thadhani R, Hakim RM. Warfarin use associates with increased risk for stroke in hemodialysis patients with atrial fibrillation. *J Am Soc Nephrol* 2009;**20**:2223–33.
9. Wizemann V, Tong L, Satayathum S, Disney A, Akiba T, Fissell RB, et al. Atrial fibrillation in hemodialysis patients: clinical features and associations with anticoagulant therapy. *Kidney Int* 2010;**77**:1098–106.
10. Herzog CA, Asinger RW, Berger AK, Charytan DM, Díez J, Hart RG, et al. Cardiovascular disease in chronic kidney disease. A clinical update from Kidney Disease: Improving Global Outcomes (KDIGO). *Kidney Int* 2011;**80**:572–86.
11. Luo G, Ducy P, McKee MD, Pinero GJ, Loyer E, Behringer RR, et al. Spontaneous calcification of arteries and cartilage in mice lacking matrix GLA protein. *Nature* 1997;**386**:78–81.
12. Ketteler M, Rothe H, Brandenburg VM, Westenfeld R. The K-factor in chronic kidney disease: biomarkers of calcification inhibition and beyond. *Nephrol Dial Transplant* 2014;**29**:1267–70.
13. Schurgers LJ, Joosen IA, Laufer EM, Chatrou ML, Herfs M, Winkens MH, et al. Vitamin K-antagonists accelerate atherosclerotic calcification and induce a vulnerable plaque phenotype. *PLoS One* 2012;**7**:e43229.
14. Wada T, McKee MD, Steitz S, Giachelli CM. Calcification of vascular smooth muscle cell cultures: inhibition by osteopontin. *Circ Res* 1999;**84**:166–78.
15. Shobeiri N, Adams MA, Holden RM. Vascular calcification in animal models of CKD: a review. *Am J Nephrol* 2010;**31**:471–81.
16. Chong CR, Chan WP, Nguyen TH, Liu S, Procter NE, Ngo DT, et al. Thioredoxin-interacting protein: pathophysiology and emerging pharmacotherapeutics in cardiovascular disease and diabetes. *Cardiovasc Drugs Ther* 2014;**28**:347–60.
17. Zitman-Gal T, Green J, Pasmanik-Chor M, Oron-Karni V, Bernheim J. Endothelial pro-atherosclerotic response to extracellular diabetic-like environment: possible role of thioredoxin-interacting protein. *Nephrol Dial Transplant* 2010;**25**:2141–9.
18. Tanaka N, Dalton N, Mao L, Rockman HA, Peterson KL, Gottshall KR, et al. Transthoracic echocardiography in models of cardiac disease in the mouse. *Circulation* 1996;**94**:1109–17.
19. London GM, Guérin AP, Marchais SJ, Métivier F, Pannier B, Adda H. Arterial media calcification in end-stage renal disease: impact on all-cause and cardiovascular mortality. *Nephrol Dial Transplant* 2003;**18**:1731–40.
20. Proudfoot D, Skepper JN, Hegyi L, Bennett MR, Shanahan CM, Weissberg PL. Apoptosis regulates human vascular calcification in vitro: evidence for initiation of vascular calcification by apoptotic bodies. *Circ Res* 2000;**87**:1055–62.
21. Shroff RC, Shanahan CM. The vascular biology of calcification. *Semin Dial* 2007;**20**:103–9.
22. Kovacic JC, Moreno P, Nabel EG, Hachinski V, Fuster V. Cellular senescence, vascular disease, and aging: part 2 of a 2-part review: clinical vascular disease in the elderly. *Circulation* 2011;**123**:1900–10.
23. London GM, Marchais SJ, Guérin AP, Métivier F. Arteriosclerosis, vascular calcifications and cardiovascular disease in uremia. *Curr Opin Nephrol Hypertens* 2005;**14**:525–31.
24. Crouthamel MH, Lau WL, Leaf EM, Chavkin NW, Wallingford MC, Peterson DF, et al. Sodium-dependent phosphate cotransporters and phosphate-induced calcification of vascular smooth muscle cells: redundant roles for PiT-1 and PiT-2. *Arterioscler Thromb Vasc Biol* 2013;**33**:2625–32.
25. Chen NX, Duan D, O'Neill KD, Wolisi GO, Koczman JJ, Laclair R, et al. The mechanisms of uremic serum-induced expression of bone matrix proteins in bovine vascular smooth muscle cells. *Kidney Int* 2006;**70**:1046–53.
26. Moe SM, Chen NX. Mechanisms of vascular calcification in chronic kidney disease. *J Am Soc Nephrol* 2008;**19**:213–6.
27. Danziger J. Vitamin K-dependent proteins, warfarin, and vascular calcification. *Clin J Am Soc Nephrol* 2008;**3**:1504–10.
28. Ketteler M, Rothe H, Krüger T, Biggar PH, Schlieper G. Mechanisms and treatment of extraosseous calcification in chronic kidney disease. *Nat Rev Nephrol* 2011;**7**:509–16.
29. Winkelmayer WC, Liu J, Setoguchi S, Choudhry NK. Effectiveness and safety of warfarin initiation in older hemodialysis patients with incident atrial fibrillation. *Clin J Am Soc Nephrol* 2011;**6**:2662–8.
30. Dahal K, Kunwar S, Rijal J, Schulman P, Lee J. Stroke, major bleeding, and mortality outcomes in warfarin users with atrial fibrillation and chronic kidney disease: a meta-analysis of observational studies. *Chest* 2016;**149**:951–9.
31. Nigwekar SU, Zhao S, Wenger J, Hymes JL, Maddux FW, Thadhani RI, et al. A nationally representative study of calcific uremic arteriolopathy risk factors. *J Am Soc Nephrol* 2016;**27**:3421–9.
32. Lee KH, Li SY, Liu JS, Huang CT, Chen YY, Lin YP, et al. Association of warfarin with congestive heart failure and peripheral artery occlusive disease in hemodialysis patients with atrial fibrillation. *J Chin Med Assoc* 2017;**80**:277–82.