INTRODUCTION

Cardiovascular complications are common among patients with chronic kidney disease (CKD), especially those with end-stage renal disease (ESRD), who undergo hemodialysis thrice a week. 2 Vascular calcification is most frequent in patients with end-stage renal disease and considered to be an independent marker of cardiovascular risk. 3, 4 Medial calcification, often named Monckeberg's arteriosclerosis, is typical in

Objective: In order to find new strategies for the prevention of vascular calcification in uremic individuals especially treated by dialysis and develop novel therapeutic targets in vascular calcification, we explore the role of KCa3.1 in alkalinization-induced VSMCs calcification in vitro.

Method: Rat VSMCs calcification model was established by beta-glycerophosphate (β-GP, 10 mM) induction. The pH of Dulbecco's modified Eagle's medium (DMEM) was adjusted every 24 h with 10 mM HCl or 10 mM NaHCO3. The mineralization was measured by Alizarin Red staining and O-cresolphthalein complex one method. mRNA and protein expression were detected by RT-PCR and Western blot or immunofluorescence. Ca2+ influx was measured by Elisa.

Result: The results indicated that alkalinization induced an increase in Ca2+ influx to enhance VSMCs calcification. Furthermore, the increase of calcification was associated with the expression of KCa3.1 via advanced expression of osteoblastic differentiation markers alkaline phosphatase (ALP) and Runx-related transcription factor 2 (Runx2). Blocking KCa3.1 with TRAM-34 or shRNA vector can significantly lowered the effects of calcification in the activity of ALP and Runx2 expression.

Conclusion: Together all, our studies suggested that alkalinization can promote vascular calcification by upregulating KCa3.1 channel and enhancing osteogenic/chondrogenic differentiation by upregulating Runx2. The specific inhibitor TRAM-34 and KCa3.1-shRNA ameliorated VSMCs calcification by downregulating KCa3.1.

Keywords: alkalinization, KCa3.1, Runx2, vascular calcification
patients with ESRD compared with CAD patients. VSMCs, as a main component of vascular media, can affect calcification process through many ways, such as trans-differentiation into bone/chondrocyte-like cells, release of matrix vesicles, and induction of apoptosis.

In uremic patients, serum bicarbonate peaks following bicarbonate loading during hemodialysis. Clinical trials have concluded that if a 40% phosphate rebound were to occur 2 h after termination of dialysis, the calculated risk of metastatic calcification would increase 2.8-fold compared to pre-dialysis conditions. Metabolic acidosis is a common complication in patients with CKD, and alkali therapy has been shown to retard the progression of CKD. An alkaline pH augments calcification of rat aortas in culture. De Solis et al studies have confirmed that alkalization increased vascular calcification in VSMCs induced by high phosphorus and in uremic rats models. This raises the possibility that the practice of alkaline loading during hemodialysis may contribute to vascular calcification. However, the specific mechanism for the alkalization to participate in the vascular calcification remains unclear.

As we know, ion channels relate to various cellular functions. In early 1990s, researches showed that in tracheal smooth muscle strips precontracted by high K+ solutions, alkalization increased [Ca2+]i. KCa channels are sensitive to intracellular Ca2+ concentration and can be classified into three distinct groups according to their conductance capacity: large-conductance (KCa1.1), intermediate-conductance (KCa3.1), and small-conductance channels (KCa2.3). KCa3.1 is widely distributed throughout the cells such as endothelia, fibroblasts, T-lymphocytes, VSMCs, and several cancer cells. The intermediate-conductance calcium-activated potassium channel KCa3.1 contributes to a variety of cell activation processes such as inflammation, carcinogenesis, and vascular remodeling.

Recently, inhibition of KCa3.1 was shown to suppress rat VSMCs calcification induced by calcified medium. Kohler, R. has proved that the switch toward KCa3.1 expression may promote excessive neointimal VSMCs proliferation. Moreover, KCa3.1 may play a novel role in human bronchial smooth muscle phenotypic modulation. In this study, we present evidence that the KCa3.1 channel is required for rat VSMCs calcification in response to alkalization.

2 | MATERIALS AND METHODS

2.1 | Cell culture and groups

Primary vascular smooth muscle cells were extracted from healthy male Sprague-Dawley rat aorta weighing 80–100 g (Hebei Medical University, Hebei province, China). Calcification was induced by β-glycerophosphate (10 mM) in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 15% fetal bovine serum and 100 U/ml penicillin and 100 μg/ml streptomycin. The pH of DMEM was adjusted every 24 h with 10 mM HCl or 10 mM NaHCO3.

In order to explore the effect of alkalization on VSMCs calcification induced by high phosphorus, VSMCs were randomly divided into 4 groups: pH7.4 normal medium group, pH7.4 calcified medium group, pH7.7 calcified medium group, and pH8.0 calcified medium group. Additionally, to investigate whether KCa3.1 is involved in regulating VSMCs calcification, cells were divided into the following 4 groups: pH8.0 normal medium group, pH8.0 calcified medium group, pH8.0 calcified medium+20 μmol/L verapamil group, and pH8.0 calcified medium+20 nmol/L TRAM-34 group. Also, to elucidate the influence of KCa3.1 specific knockdown on the production of calcification cells was randomly divided into 4 groups: pH8.0 normal medium group, pH8.0 calcified medium group, pH8.0 calcified medium group+control vector transfection group, and pH8.0 calcified medium group+KCa3.1-shRNA vector transfection group.

2.2 | Aortic calcification in vitro

After anesthesia of 6 week-old SD male rats, the thoracic aortas were removed under aseptic condition, and the vessels were cut into 2–3 mm rings and placed in DMEM medium containing 10% fetal bovine serum. The calcification was induced by β-glycerophosphate (10 mM), and the PH was adjusted by NaHCO3. The vascular rings were randomly divided into groups which were consistent with that of cell cultured in vitro.

2.3 | von Kossa staining

Rats aortic segments were fixed in 4% paraformaldehyde at room temperature for 24 h and embedded in paraffin. Aortic samples were cut into 4 μm thick sections that were then prepared for von Kossa staining. After deparaffinized, the tissues were stained with 5% silver nitrate by exposure to daylight for 45 min. Microphotographs were taken using identical exposure conditions with a Nikon 995 camera.

2.4 | Immunohistochemistry

Aortic tissues were deparaffined, and epitopes were retrieved by boiling the tissues in sodium citrate (PH 6.0) for 3 min with pressure cooker. The sections were then blocked with goat serum for 1 h and incubated with primary antibodies overnight at 4°C, followed by the incubations of biotinylated secondary antibody and HRP-conjugated streptavidin on the following day. The primary antibodies used were as follows: KCa3.1 (1:100) and Runx2 (1:70). Staining was developed with diaminobenzidine (DAB) before counterstaining with hematoxylin. The primary antibodies were obtained from Abcam Company (Cambridge, MA, USA). The sections were imaged with Nikon 995 camera and integrated optical density (IOD) of positive region was analyzed quantitatively using Image Pro-Plus 5.0 software (Media Cybernetics, Silver Spring, MD).

2.5 | Assay of alkaline phosphatase (ALP) activity

The cells were cultured for 12 days and washed 3 times with PBS. The protein assay was performed with the bicinchoninic acid protein
assay reagent (Beijing Solarbio Science & Technology Company Co., Ltd.). ALP activity was measured by Alkaline Phosphatase Activity Detection kit (Nanjing Jiancheng Bioengineering Institute). Each value was normalized relative to the protein concentration of the same culture.

2.6 | Calcification assays

The calcium content was determined by spectrophotometry, using a calcium assay kit (BioSino Biotechnology). The results were then normalized by protein content, which was quantified by the BCA protein assay kit (Beijing Solarbio Science & Technology Company Co., Ltd.).

2.7 | Intracellular Ca2+ Measurement

After cultured for 4 days, VSMCs were put in black 96-well microtiter plates (Greiner Bio-One, Frickenhausen, Germany). After 30 min preincubation at 37°C with the Fluo 3-AM and probenecid (Fluo-4 NW Calcium Assay Kit; Molecular Probes), VSMCs were treated with 80 mmol/L K+ as promoter and free intracellular Ca2+ levels were measured using Elisa (CYTATION3, Bio Tek) by UV absorbance at 526 nm.

2.8 | PCR

Total RNA was isolated by trizol reagent (Invitrogen), and cDNA was transcribed from total RNA using the RT-for-PCR kit (Clontech). cDNA was used as regular RT-PCR template. The primer sequences were list in Table 1. The products were tested in 2% agarose gel in electrophoresis. The pictures were analyzed by Gel Documentation System (CST Biological Reagents Company Limited), and the final data were expressed as the mRNA level relative to that of GAPDH.

2.9 | Western blot analysis

After stimulation for 4 days, total proteins were extracted from the VSMCs, and the concentrations were measured with the BCA protein assay kit. The samples were mixed with loading buffer and boiled for 5 min. The protein samples were resolved on 10% SDS-PAGE gel (70 V, 120 min) and electro-transferred to a PVDF membrane (15 V, 30 min) and blocked with 5% non-fat dry milk in TBS-T [20 mmol/L Tris-HCl (pH 7.6), 150 mmol/L NaCl, and 0.02% Tween 20] (Invitrogen, Carlsbad, CA, USA) for 1 h at room temperature with agitation. Subsequently, the primary antibodies of Kca3.1 (1:300), Runx2(1:200), and GAPDH (1:500) were added to the samples, respectively, and incubated at 4°C overnight. After 1 h incubation with the secondary antibody (1:2000, Jackson ImmunoResearch), the samples were visualized using ECL reagent (Pierce) and imaged. The images were analyzed by using Chemiluminescence detection System. GAPDH was used as an endogenous control. The experiments were replicated three times.

2.10 | Cell transfection

The shRNA plasmid targeting the rat KCa3.1 gene was obtained from Hanbio Biotechnology, and the target sequences were 5’-GCACCUUUCAGACACACU-3’. VSMCs were transfected with either KCa3.1-shRNA or negative control using Lipofectamine 3000 (Invitrogen) according to the manufacturer’s instruction.

2.11 | Statistical analysis

Results were presented as means ± SD, and SPSS 17.0 software (SPSS Company) was used for the ANOVA analysis and Dunnett test. For all the statistical tests, p < 0.05 was defined as statistically significant difference.

3 | RESULTS

3.1 | Alkalinitization promotes the calcification process in Aortic rings

After stimulation for 12 days, we examined the effects of alkalinitization on the calcification of aortic rings induced by β-GP via von Kossa staining and Ca accumulation. Alkalinitization significantly augmented calcium deposition in aortic rings induced by β-GP.
using von Kossa staining (Figure 1A). What is more, quantitative analysis showed that the calcium content of the aortic rings in the PH7.7 calcified group increased 1.36 times and the PH8.0 calcified group increased 2.02 times compared with the PH7.4 calcified group, and with the increasing of alkalinization concentration, the degree of aortic calcification increased (Figure 1B). These results suggested that alkalinization played a catalytic role in the induction of calcification.

The results of cell experiments in vitro were consistent with those of aortic rings. Alizarin red staining indicated that the calcium salt deposition in the PH8.0 calcified group was significantly higher than that in the PH7.4 calcified group (Figure 1C). Furthermore, we found that alkaline increased the calcium content of the VSMCs in a concentration-dependent manner, as demonstrated by the

Calcification assays. The result showed that calcium content was 23.3% and 69.6% higher, in PH7.7 calcified group and PH8.0 calcified group than that of PH7.4 calcified group, respectively (Figure 1D).

3.2 Alkalinization promotes Ca\textsuperscript{2+} influx in VSMCs

We tested the possibility whether stimulation by alkalinization-induced intracellular Ca\textsuperscript{2+} fluxes. The results of immunofluorescence showed that compared with the normal control group, the fluorescence intensity induced by β-glycerophosphate was significantly increased, and the fluorescence intensity increased gradually with the increase of PH which corresponded to the release of intracellular Ca\textsuperscript{2+} fluxes (Figure 1E and F).
3.3 Effect of Alkalinization on KCa3.1 and Runx2 expression and ALP activity in VSMCs

We first explored the expression changes of KCa3.1 related to osteogenic transcription factor Runx2. RT-PCR and Western blot were performed to demonstrate marked induction of KCa3.1 and Runx2 in rat VSMCs which were grown in normal and calcified medium in different extracellular PH (7.4, 7.7, and 8.0) for 4 days. The expression of KCa3.1 and Runx2 was significantly higher in the β-glycerophosphate induced group than in the control group. Moreover, the expression of KCa3.1 and Runx2 was upregulated as extracellular pH values increased. Then, we tested the effect of alkalinization on ALP activity. Compared with PH7.4 control group, the addition of alkalinization triggered an increase in ALP activity in PH7.4 calcified group and PH8.0 calcified group by 36.4% and 112.4%, respectively.
Thus, under the appropriate conditions, VSMCs in culture can recapitulate the expression profile of osteogenic transcription factor.

3.4 | Effect of Alkalination on KCa3.1 and Runx2 expression in aortic rings

To determine whether the expression of KCa3.1 and Runx2 was altered in advance, immunostaining was used in control group and in different pH group aortas from rats cultured with β-glycerophosphate media. The expression of KCa3.1 and Runx2 was significantly increased in the calcified groups compared with the control group. Besides, the expression of KCa3.1 and Runx2 was increased as extracellular pH increasing (Figure 2D).

3.5 | TRAM-34 attenuates calcification in VSMCs

To prove intracellular Ca\(^{2+}\) playing an important role in activating KCa3.1 and calcification, we added verapamil to block Ca\(^{2+}\) influx in DMEM. To provide direct evidence that KCa3.1 channel was indeed involved in Runx2 proteins expression, VSMCs were then treated with TRAM-34(KCa3.1 blocker). Verapamil and TRAM-34 induced a decrease in calcium deposition by alizarin red staining and calcium content (Figure 3A and B).

3.6 | TRAM-34 attenuates Ca\(^{2+}\) influx in VSMCs

The results of Fluo-3AM show that the administration of verapamil or TRAM-34 can block the uptake of calcium in the VSMCs induced by alkalination as compared to the pH 8.0 calcified group (Figure 3C and D).

3.7 | TRAM-34 attenuates Runx2 expression and ALP activity in VSMCs

PCR results showed that after administration of the KCa3.1 inhibitor verapamil or TRAM-34, mRNA level of Runx2 decreased by 21.9% and 21.2%, respectively, compared with PH8.0 calcified group (Figure 4A). The expression of Runx2 protein decreased by 30.7% and 31.3%, respectively, in accordance with PCR results (Figure 4B). The results of Figure 4C also demonstrated that inhibition of KCa3.1 decreased intracellular ALP activity. All above results suggested that blocking KCa3.1 could downregulate the osteogenic transcription of VSMCs.
3.8 | TRAM-34 attenuates calcification and Runx2 expression in aortic rings

The results of immunohistochemistry confirmed that the Runx2 positive area in verapamil or TRAM-34 group was lower than that in PH8.0 control group (Figure 4D). To prove the effect of TRAM-34 on calcification in advanced, calcium deposition was measured by von Kossa staining and calcium content (Figure 4E and F). After stimulated with Verapamil and TRAM-34, calcium deposition was significantly decreased compared with pH8.0 calcified group.

3.9 | Specific knockdown KCa3.1 can reduce Runx2 expression and ameliorate calcification in VSMCs

To further demonstrate KCa3.1 channel does regulate the expression of Runx2 proteins and affects cell calcification, KCa3.1 specific knockdown plasmid was transfected into VSMCs. Western blot result indicated that the Runx2 expression decreased by 22.4% after transfected with KCa3.1-shRNA, compared with the negative control group. The Alizarin red staining also demonstrated that down
expression of KCa3.1 led to a lower amount of dye incorporation (Figure 5B), compared with control group.

All of the above results indicated that downregulation of KCa3.1 can ameliorate calcification caused by alkalinization-induced transformation of the VSMCs into osteoblasts via decreased Runx2.

4 | DISCUSSION

Recently, studies have concentrated on the effect of alkalinization which is particularly important in hemodialysis patients, since frequently exposed to alkaline loading during hemodialysis and supplied with alkali during pre-hemodialysis on media calcification. However, the molecular mechanism is still unclear. With high extracellular concentrations of phosphate medium, cell culture is an important method to explore the mechanism of calcification. Both alizarin red staining and Ca accumulation showed markedly increased VSMCs calcification induced with β-glycerophosphate medium whose pH was approached to 7.4, 7.7, and 8.0. Similarly, alkalinization-related vascular calcification data obtained in cultured aortic rings.

KCa3.1 mediates cellular calcification of many cell types including VSMCs. The protection to vascular calcification of KCa3.1 blocker is found in murine VSMCs. However, the mechanism of KCa3.1 in alkalinization-induced VSMC calcification is unknown. In our study, we provided evidence that intermediate-conductance Ca\(^{2+}\)-dependent K\(^+\) channel (KCa3.1) played a critical role in alkalinization-induced vascular calcification with a calcified medium containing elevated levels of β-glycerophosphate.

We found that increased expression of KCa3.1 was associated with increased vascular calcification in the alkalinization-induced VSMCs. KCa3.1 channel have been demonstrated to promote mitogenesis in vascular smooth muscle (VSM) cells and play a pivotal role in disease states characterized by excessive cell proliferation. Using multiple molecular biology approaches, we confirmed that alkalinization-induced vascular calcification upregulated KCa3.1 gene and protein expression. In addition, vascular calcification was abolished by the KCa3.1 channel blocker. These results demonstrated a direct link between KCa3.1 channels and vascular calcification.

In lymphocytes and fibroblasts, KCa3.1 channel enhanced the electrochemical driving force for Ca\(^{2+}\) influx through membrane hyperpolarization. Increased intracellular Ca\(^{2+}\) concentration contributes to gene transcription. Higher Ca\(^{2+}\) affinity of KCa3.1 channel would result in channel opening. The resultant K\(^+\) efflux causes membrane hyperpolarization in response to subtle increases in the intracellular Ca\(^{2+}\) concentration. That may be the reason that KCa3.1 channel upregulated in shaping Ca\(^{2+}\) signals of calcification VSMCs.

**FIGURE 5** Transfected with KCa3.1-shRNA reduced Runx2 expression and ameliorate calcification in VSMCs. (A) Western blot detection and statistical analyses for KCa3.1 and Runx2 of VSMCs of four groups. The data were expressed as the means ± SD. *p < 0.01 versus pH8.0 calcified medium group. (n = 3). (B) Alizarin red staining at day 12 in the presence of pH8.0 normal medium group, pH8.0 calcified medium group, pH8.0 calcified medium +negative control group, and pH8.0 calcified medium +KCa3.1-shRNA group, respectively. (n = 3)
TRAM-34, the selective inhibitor of the KCa3.1, is regarded as a novel therapeutic option for different disease states. TRAM-34 was shown to suppress proliferation of VSMCs\(^\text{25}\) and occlusions after angioplasty in rats\(^\text{24}\) and to reduce infarction and angiogenesis.\(^\text{32}\) In our study, we proved that TRAM-34 protected VSMCs from calcification by blocking KCa3.1 channel and suppressing Ca\(^{2+}\) influx. After treatment with TRAM-34, uptake of Ca\(^{2+}\) was suppressed. Thus, blockade of KCa3.1 by TRAM-34 might reduce membrane hyperpolarization with subsequent reduced Ca\(^{2+}\) entry into VSMCs.

Vascular calcification is a complex regulated process including stimulation of osteogenic/chondrogenic differentiation and apoptosis.\(^\text{6,33}\) We here studied effects of the KCa3.1 on alkalinization-induced transition of VSMCs.

The process of transition involves the production of calcification promoting transcription factors and proteins such as Runx2 or osterix and a concurrent decline in the expression of calcification inhibitors, such as MGP or SM22\(^\alpha\).\(^\text{6,34,35}\) Alkalinization promoted the \(\beta\)-glycerophosphate-induced upregulation of mRNA expression of ALP and Runx2 in VSMCs. This indicates that alkalinization activates with the onset of the transition of the contractile VSMCs.

5 | CONCLUSION

In summary, our study suggests alkalinization promotes vascular calcification by upregulating KCa3.1 channel by enhancing osteogenic/chondrogenic differentiation and apoptosis. The specific inhibitor TRAM-34 protects vessels from calcification. These results offer a new strategy to prevent vascular calcification, with special reference to those treated by dialysis. This information has the potential to influence everyday decision-making in dialysate alkali content, the use of oral sodium bicarbonate and provide a rationale for further vascular studies with TRAM-34 or other potassium channel inhibitors.

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CONFLICT OF INTEREST

None of the authors disclose any financial, consulting, and personal relationships with other people or organizations that could influence the author’s work.

DATA AVAILABILITY STATEMENT

The data used to support the findings of this study are available from the corresponding author upon request.

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