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# Role of Wnt/β-Catenin Pathway in the Arterial Medial Calcification and Its Effect on the OPG/RANKL System

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**Summary**: In this study, the hypothesis that Wnt/β-catenin pathway is involved in the arterial calcification by regulating the osteoprotegerin (OPG)/receptor activator of NF-κB ligand (RANKL) system was tested. The β-catenin expression was measured in the warfarin-induced calcified arteries and the osteoblast-like cells differentiating from smooth muscle cells (SMCs) by immunohistochemistry and Western blotting. The Wnt/β-catenin pathway was activated or inhibited by lithium chloride (LiCl) or dickkopf 1 (DKK1) *in vitro* and *in vivo*. Then the calcification level was determined by von Kossa staining, $Ca^{2+}$ content assay, and alkaline phosphatase (ALP) activity assay. The expression levels of osteocalcin, OPG and RANKL were detected by Western blotting or real-time PCR. The results showed that in calcified arteries and OBL cells, the activation of Wnt/β-catenin pathway significantly enhanced the calcification as evidenced by increased von Kossa stains, $Ca^{2+}$ contents, ALP activities, and osteocalcin expression levels ($P$<0.05), and it promoted the RANKL expression ($P$<0.05), but slightly affected the OPG expression. These results indicated that the activation of Wnt/β-catenin pathway worsens the arterial calcification, probably by promoting the RANKL expression.
**Key words**: arterial calcification; Wnt/β-catenin pathway; osteoprotegerin; receptor activator of NF-κB ligand

Arterial medial calcification is a very common pathological phenomenon existing in the aging, diabetes, and chronic kidney disease[1]. It has been revealed to be attributed to many factors, such as the apoptosis of smooth muscle cells (SMCs), the formation of the matrix vesicle, the abnormality of the calcium and phosphorus metabolism, the increased expression of the inflammatory factors and bone morphogenetic proteins (BMPs), and the decreased expression of osteopontin, matrix G protein (MGP), and fetoprotein[2–4]. In recent years, many studies demonstrated that the arterial calcification was not only passive deposition of calcium and phosphorus in the artery, but a highly active regulated process like the bone formation[5, 6]. There are osteoblast-like (OBL) cells in the artery, which differentiate from SMCs of the artery and can induce the calcium deposition just like the osteoblasts[7, 8].

The Wnt/β-catenin pathway is an important signaling pathway that mainly regulates the cell differentiation[9]. It was found that the Wnt/β-catenin pathway facilitated the bone formation via promoting the osteoblast differentiation[10, 11], and recent studies showed that the Wnt/β-catenin pathway also had relevance to the OBL cells differentiation in the arterial calcification. However, the precise role of Wnt/β-catenin pathway in the arterial calcification remains unclear. In addition, it has been proven that some regulation systems, such as osteoprotegerin (OPG)/receptor activator of NF-κB ligand (RANKL), which is the key regulation system that controls the bone formation[12, 13], are also involved in the arterial calcification[14, 15]. Studies have shown that RANKL promotes the calcification[16, 17], and OPG inhibits the calcification[18, 19]. The ratio of OPG/RANKL is related to the development of the arterial calcification. Therefore, in the current study, we aimed to examine the activity of Wnt/β-catenin pathway, the change of

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calcification level, and the expression of OPG and RANKL in the calcified arteries and SMCs after use of the activator lithium chloride (LiCl) and inhibitor dickkopf 1 (DKK1) of the Wnt/β-catenin pathway in order to shed light on the exact role of Wnt/β-catenin pathway in the arterial calcification.

## 1 MATERIALS AND METHODS

The antibodies of osteocalcin, Wnt1, Wnt2, Wnt3a, Wnt4, Wnt5a, Wnt5b, Wnt7a, Wnt7b, Wnt8b, Wnt10b, and the alkaline phosphatase (ALP) activity assay kit were bought from Bioss (China). The antibodies of OPG, RANKL, β-catenin, and DKK1 were procured from R and D Systems (USA). Warfarin, LiCl, vitamin K1, vitamin C, and β-glycerophosphate were obtained from XinYi Medical Company (China). Atorvastatin was bought from Pfizer (China). Sprague Dawley (SD) rats were from the Animal Center of Tongji Medical College, China. The PCR primers of OPG and RANKL were provided by ProMab (China).

### 1.1 Establishment of Animal Model and Grouping

The arteries in 6-week-old healthy SD male rats of 180–200 g weight were induced to cause calcification with warfarin as previously described[20, 21]. One hundred SD rats were randomly assigned to 5 groups: normal group, calcified group, atorvastatin treated group, normal + LiCl treated group, and calcified + LiCl treated group. Each group was divided into early and late subgroups ($n$=10 per subgroup), and all the rats were fed on a normal diet. First, all the rats were injected with vitamin $K_1$ (XinYi, China) subcutaneously at 10 $mg \cdot kg^{-1} \cdot day^{-1}$ for one week (qd). Then, rats in the normal group were intragastrically administered with normal saline; those in the calcified group were intragastrically treated with 15 $mg \cdot kg^{-1} \cdot day^{-1}$ warfarin (qd, XinYi, China); those in the atorvastatin treated group received intragastric administration of both 15 $mg \cdot kg^{-1} \cdot day^{-1}$ warfarin and 20 $mg \cdot kg^{-1} \cdot day^{-1}$ atorvastatin (Pfizer, USA) once a day; some rats in the normal + LiCl treated group received intragastric treatment of 150 $mg \cdot kg^{-1} \cdot day^{-1}$ LiCl (XinYi, China) and the others were given 300 $mg \cdot kg^{-1} \cdot day^{-1}$ LiCl (qd); rats in the calcified + LiCl treated group were intragastrically administered with both warfarin at 15 $mg \cdot kg^{-1} \cdot day^{-1}$ and LiCl at 150 $mg \cdot kg^{-1} \cdot day^{-1}$ (qd). All the rats in the early subgroup were sacrificed with 20% chloral hydrate at 0.7 $mL \cdot (100\ g)^{-1}$ at 17 days, and those in the late subgroup at 34 days. Their arteries were collected for the following measurements.

### 1.2 Cell Culture and Grouping

Rat artery SMCs were obtained and cultured as previously described[22]. They were induced to differentiate into OBL cells by β-glycerophosphate, and Wnt/β-catenin pathway was activated with LiCl in the cells[23, 24]. The cells were divided randomly into 6 groups: SMC group, OBL group, atorvastatin treated group, SMC + LiCl treated group, OBL + LiCl treated group, OBL + DKK1 treated group. Each group was also divided into 2 subgroups. i.e. early subgroup and late subgroup. In the OBL group, 10 mmol/L β-glycerophosphate (XinYi, China) and 50 μg/mL vitamin C (XinYi, China) were added; In atorvastatin treated group, 10 mmol/L β-glycerophosphate, 50 μg/mL vitamin C and 1 μmol/L atorvastatin were given; In the SMC + LiCl treated group, SMCs were treated with 10 or 20 mmol/L LiCl; in the OBL + LiCL treated group, 10 mmol/L β-glycerophosphate, 50 μg/mL vitamin C and 10 mmol/L LiCl were added; in the OBL + DKK1 treated group, 10 mmol/L β-glycerophosphate, 50 μg/mL vitamin C and 0.1 μg/mL DKK1 (R & D, USA) were used. The cells were collected at 7 days in the early subgroups and at 14 days in the late subgroups.

### 1.3 Assay of the Calcification Level

**1.3.1 von Kossa Staining** The arteries were fixed in 4% paraformaldehyde, underwent gradient dehydration with alcohol and paraffin embedding, and then were cut into slices of 5 μm, which were later dewaxed and rehydrated. For cells, they were cultured in the 6-cell plate. The samples including the arteries and cells were washed with deionized water three times, incubated with 1% silver nitrate solution, exposed in sunlight for 60 min, and then incubated in 5% sodium thiosulfate solution for 5 min to remove unreacted silver. They were washed with deionized water three times, and counterstained with 0.2% nuclear fast red solution. Finally, the stained slides were dehydrated and mounted with DPX mountant. The black stains were indicative of calcium deposition.

**1.3.2 Calcium Content Detection** Approximately 1-cm thoracic arteries were collected, dried at 70°C for 24 h and weighed. They were treated with 2 mol/L concentrated nitric acid at room temperature for 24 h. Thereafter, they were heated on an automatic electric heater until all the nitric acid was volatilized. After the samples had cooled down, 2 mL deionized water containing 27 nmol/L KCl and 27 μmol/L $LaCl_3$ was added. Subsequently, approximately 150 μL of 1% strontium chloride was given. Finally, an atomic absorption spectrophotometer (SpectrAA-240FS, USA) was used to detect the calcium content in the arteries of all groups. The results were expressed as mg/g.

The OBL cells were collected and rinsed with PBS, and then decalcified with 0.6 mmol/L HCl at 4°C overnight. After removal of the supernatants, cells were treated with lysis buffer containing 0.1 mmol/L NaOH and 0.1% sodium dodecyl sulfate (SDS). Afterwards, the levels of calcium released from cell cultures were determined colorimetrically via the o-cresolphthalein complexone method (calcium kit 587-A, Sigma, USA). Finally, the calcium levels were normalized to the cellular protein content of the culture and expressed as

μg/mg cellular protein.

**1.3.3 ALP Activity Assay** After the arteries were homogenated in homogenized buffer (20 mmol/L HEPES containing 0.2% NP-40 and 20 mmol/L $MgCl_2$), they were centrifuged at 8 000× *g* for 10 min. For cells, SMCs were washed with PBS and lysed by ultrasonic wave (40 W) in icy water for 5 min. The protein contents of the supernatants from arteries and SMCs were determined using the Bradford method. Afterwards, the samples were incubated in 0.1 mol/L $NaHCO_3$-$Na_2CO_3$ buffer (pH=10.0) containing 2 nmol/L $MgCl_2$ and 8 nmol/L p-nitrophenol phosphate (pNPP) (Bioss, China) for 30 min at 37°C. The reaction was stopped by adding 0.4 mol/L NaOH. The amount of p-nitrophenol released by the reaction was measured with a spectrophotometer at 405 nm. The ALP activity was normalized by the total protein content.

**1.3.4 Western Blot Analysis of Osteocalcin Expression** The samples were washed three times in cold PBS and lysed for 30 min at 4°C in the buffer containing 1% Nonidet P-40, 50 mmol/L Tris-HCl (pH=7.5), 100 mmol/L NaCl, 5 mmol/L EDTA and 1 mmol/L phenylmethylsulfonyl fluoride. Supernatant was collected after centrifugation at 13 000 *g* at 4°C for 10 min. The protein content of samples was determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, USA). Subsequently, supernatant samples containing 20 μg of protein were subjected to electrophoresis. After the supernatant samples had been loaded onto a 10% SDS-polyacrylamide gel and run at 200 V for 45 min, the gel was transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech, UK) and then gently shaken for 2 h using 5% nonfat dried milk in TBS [50 mmol/L Tris-HCL and 0.15 mol/L NaCl (pH 7.5)] containing 0.1% Tween 20. The membrane was washed with TBS and incubated with the primary antibody (1:400, R&D Systems, USA) at a temperature of 4°C overnight. In the following day, the membrane was washed with TBS three times (for a period of 5 min each time) so as to remove non-specific primary antibody binding. Afterwards, the membrane was incubated with secondary antibody and horseradish peroxidase (1:40 000, USA) for 1 h at room temperature. It was then washed three times (for a period of 10 min each time) and detected with the enhanced chemiluminescence system (Amersham Pharmacia Biotech, USA). For the internal control, the antibody against glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (1:800, SANTA, USA) was used. Filters were subsequently exposed to Kodak Bio Max Light-1 films, with the intensity of Western blot signals quantified via densitometry.

**1.4 Assay of β-catenin in the Arteries and SMCs**

**1.4.1 Immunohistochemical Method** The samples of arteries and SMCs were prepared. The internal peroxidase activity was quenched with 1% hydrogen peroxide. The samples were placed in PBS containing blocking serum for nonspecific immunoglobulin sites, and then incubated with the first antibody (1:200, anti-rat β-catenin antibody, R and D Systems, USA) overnight at 4°C. After washes 3 times in PBS, samples were incubated with biotinylated secondary antibody (Bioss, China) and streptavidin-horseradish (HRP) (Bioss, China). The HRP product was developed with a liquid 3, 3′-diaminobenzidine-positive substrate-chromogen system. Lastly, the nucleus was counterstained with hematoxylin and sections were mounted. In negative controls, the primary antibody was replaced by PBS.

**1.4.2 Western Blot Analysis of β-catenin** The expression level of β-catenin was detected by Western blotting. The procedures were the same as those described before except that the primary antibody was replaced by β-catenin (1:400, R&D Systems, USA).

**1.5 Real-time PCR Analysis of OPG and RANKL**

Total RNA of the samples was isolated by Trizol reagent (Invitrogen, USA). To avoid DNA contamination, DNase I (Life Technologies, France) treatment was performed. The cDNA was subsequently synthesized using a Revert cDNA Synthesis kit (Fermentas, Lithuania) and then real-time reverse transcriptase polymerase chain reaction (RT-PCR) was performed in a 25 μL reaction volume using ABI 7900 Sequence Detection System (Applied Biosystems, USA). Probes and primers were designed using Primer Express software: OPG-forward 5′-ATCGGCCACGCGAACCTCAC-3′, OPG-reverse 5′-GCTGCTCGCTGGGTTTGCAG-3′; RANKL-forward 5′-AGCCTTTCAAGGGGCCGTGC-3′, RANKL-reverse 5′-GGGCCACATCGAGCCACGAA-3′; GAPDH-forward 5′-CTCATGACCACAGTCCATGC-3′, GAPDH-reverse 5′-TTCAGCTCTGGGATGACCTT-3′. Fluorescent signals were acquired at the last step of each cycle, and a melting curve was calculated at the end of the each cycles. The mRNA levels of the housekeeping gene GAPDH were measured and utilized as the internal control. The relative expression of mRNA was calculated using the comparative threshold cycle (Ct) method, and the mean value was set using the $2^{-\Delta\Delta Ct}$ method.

**1.6 Detection of the Expression of Wnt Proteins in the Arteries and SMCs**

In the animal and cell models, in the normal and calcified groups, the arteries and cells were also examined for the expression of the Wnt1, Wnt2, Wnt3a, Wnt4, Wnt5a, Wnt5b, Wnt7a, Wnt7b, Wnt8b and Wnt10b proteins by Western Blotting.

**1.7 Statistical Analysis**

The measurement data were presented as $\bar{x}\pm s$ and analyzed by *t* test or one way ANOVA. The numeration data were analyzed by Chi-Square test. *P* values less than 0.05 were considered to be significantly different.

## 2 RESULTS

### 2.1 Activated Wnt/β-Catenin Pathway Is Positively Correlated with Arterial Calcification and OBL Cell Differentiation

The Wnt/β-catenin pathway was not activated in the normal arteries or SMCs as evidenced by low expression of β-catenin in the normal groups. Studies have proved that atorvastatin inhibited the calcification[25]. In the atorvastatin treated groups, the arteries or SMCs were slightly calcified and the Wnt/β-catenin pathway was slightly activated (both $P<0.05$). They were obviously calcified and the Wnt/β-catenin pathway was profoundly activated in the calcified groups (both $P<0.05$). The activity of the Wnt/β-catenin pathway was more robust in the late subgroups than in the early subgroups (all $P<0.05$) (fig. 1, fig. 2). Furthermore, the expression levels of Wnt3a, Wnt5a, Wnt7a, and Wnt7b proteins were upregulated in calcified arteries and those of Wn3a and Wnt7a proteins were also found to increase in OBL cells (all $P<0.05$) (fig. 3).

### 2.2 Activated Wnt/β-Catenin Pathway Promotes the Arterial Calcification and the OBL Cells Differentiation

The level of ALP activity in the early subgroup, the osteocalcin expression in the late subgroup, the von Kossa stains, and the calcium content were significantly increased in the LiCl-treated calcified arteries and OBL cells as compared with those in the calcified arteries or OBL cells without treatment of LiCl (all $P<0.05$), which was especially distinct in the early subgroups (fig. 4, fig. 5, table 1). However, the treatment with LiCl alone did not induce the arterial calcification or OBL cells differentiation, but caused a slight increase in the expression of ALP, OPG, and RANKL, with no

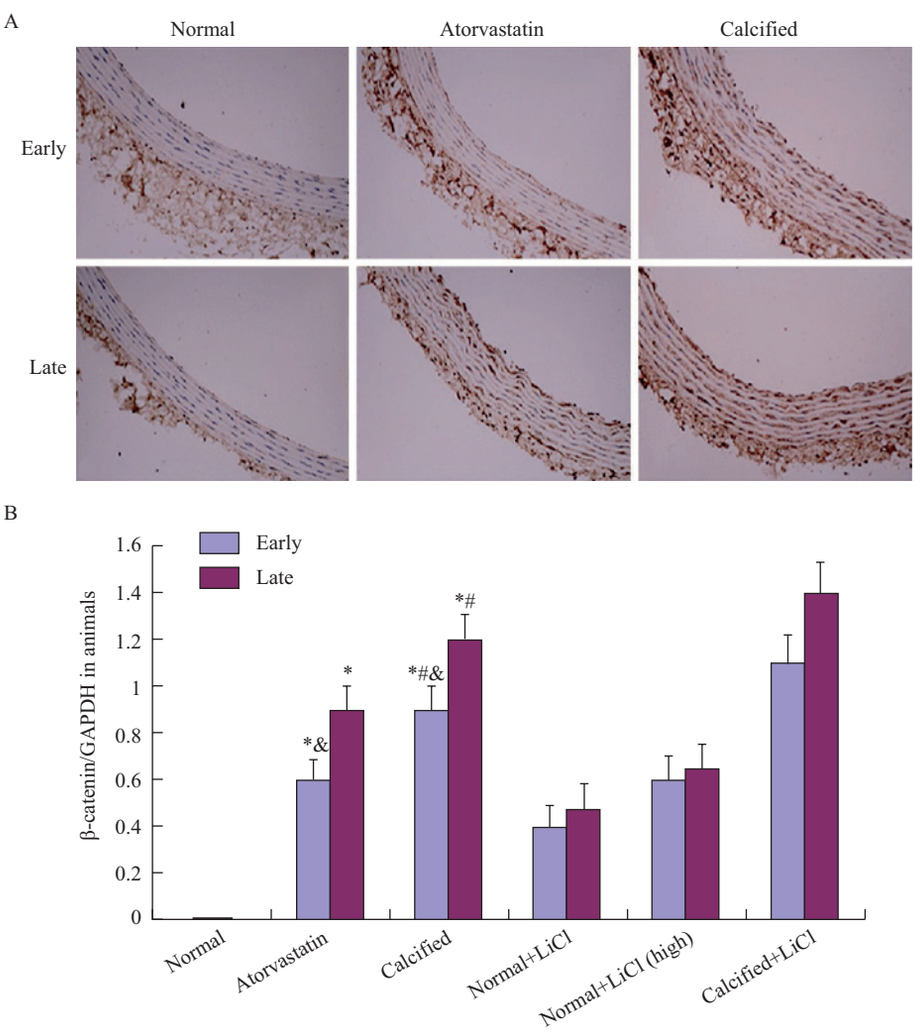


**Fig. 1** Activation of Wnt/β-catenin pathway in the calcified arteries

A: immunohistochemical analysis of the expression of β-catenin in the arteries (×400); B: Western blot analysis of the expression of β-catenin. Wnt/β-catenin pathway was activated in the calcified arteries. When calcified arteries were treated with atorvastatin, Wnt/β-catenin pathway was inhibited along with the decrease of the calcification. Wnt/β-catenin pathway was significantly activated, along with the increase of the calcification in the late subgroups as compared with the early subgroups. $^{*}P<0.05$ *vs.* normal group; $^{\#}P<0.05$ *vs.* atorvastatin group; $^{\&}P<0.05$ *vs.* late subgroup

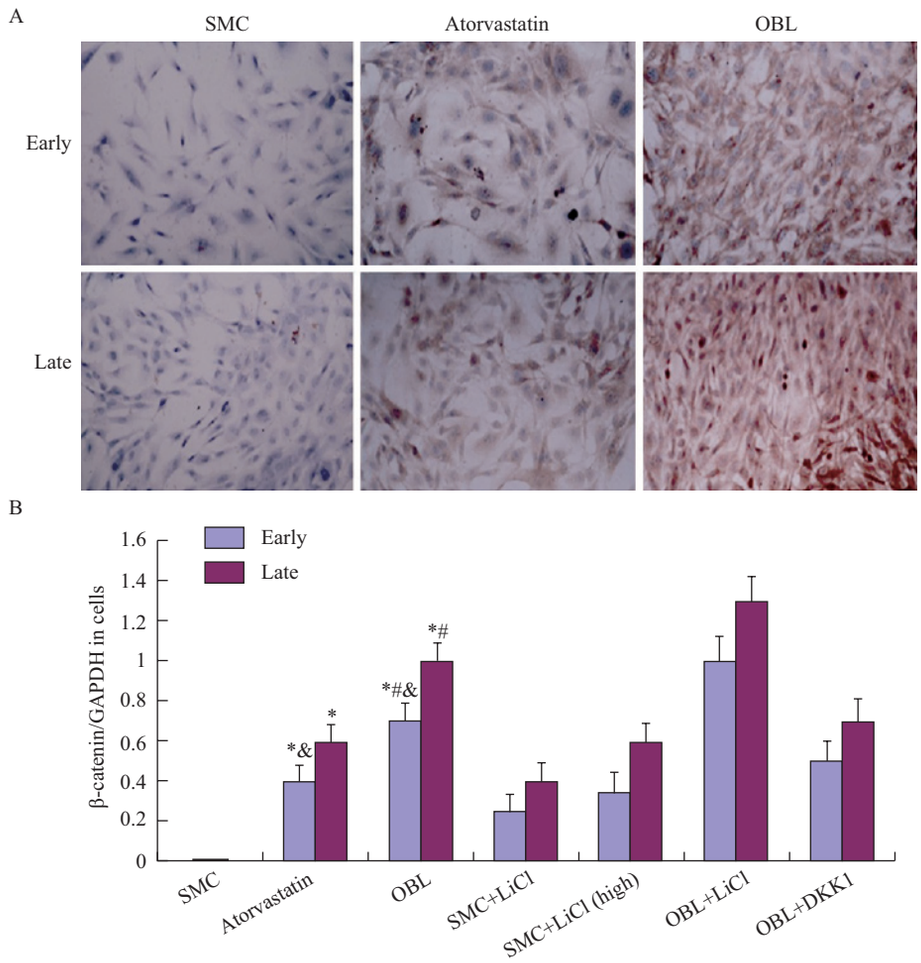


**Fig. 2** The activity of Wnt/β-catenin pathway in the OBL cells and SMCs

A: immunohistochemical analysis of β-catenin in the OBL cells and SMCs (×400); B: Western blot analysis of β-catenin in the OBL cells and SMCs. As compared to SMCs, the Wnt/β-catenin pathway in the OBL cells was activated. It was inhibited after treatment with atorvastatin. The Wnt/β-catenin pathway was significantly activated along with the increase of the calcification in the late subgroups as compared to the early subgroups. Results were obtained from three independent experiments. $^{*}P<0.05$ *vs.* SMC group; $^{\#}P<0.05$ *vs.* atorvastatin group; $^{\&}P<0.05$ *vs.* late subgroup

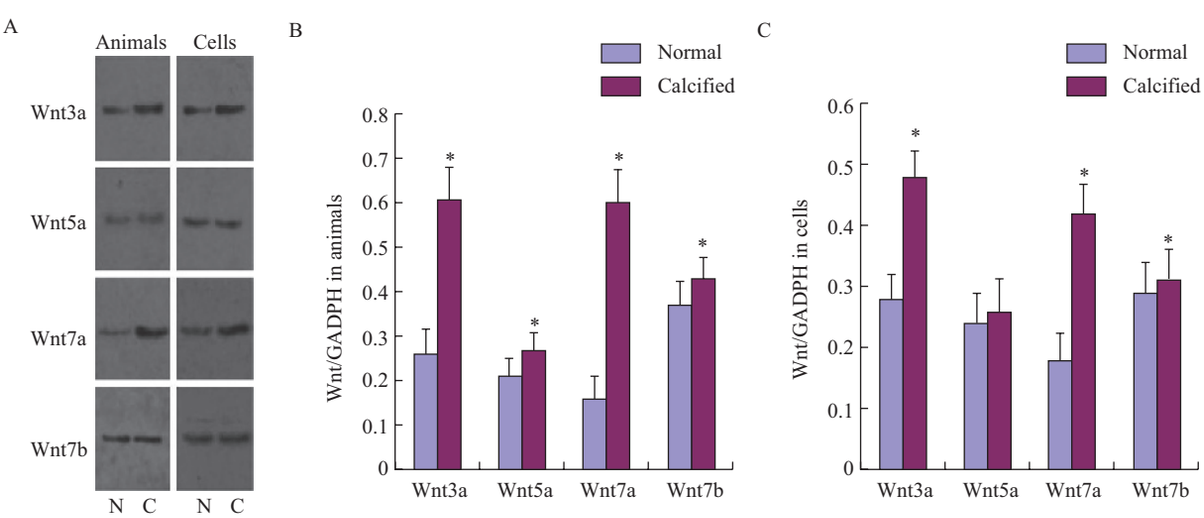


**Fig. 3** The expression of Wnt proteins in the arteries and SMCs

A: Western blots of Wnt3a, Wnt5a, Wnt7a and Wnt7b in the arteries or SMCs; B, C: statistical analysis of the expression levels of Wnt3a, Wnt5a, Wnt7a and Wnt7b in the arteries (B) or cells (C). The expression levels of Wnt3a, Wnt5a, Wnt7a, Wnt7b proteins were significantly upregulated in the calcified arteries and OBL cells (all $P<0.05$). $^{*}P<0.05$ *vs.* normal group. N: normal; C: calcified

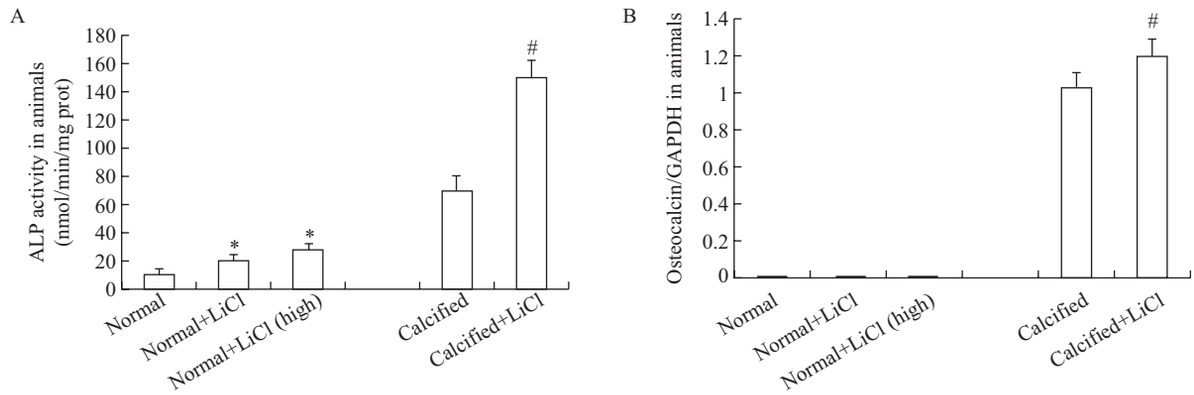


**Fig. 4** The impact of Wnt/β-catenin pathway on arterial calcification

A: The ALP activity was slightly increased in normal rats treated with LiCl but markedly increased in calcified rats treated with LiCl; B: The expression of osteocalcin was significantly increased in calcified rats treated with LiCl. $^{*}P<0.05$ *vs.* normal group, $^{\#}P<0.05$ *vs.* calcified group

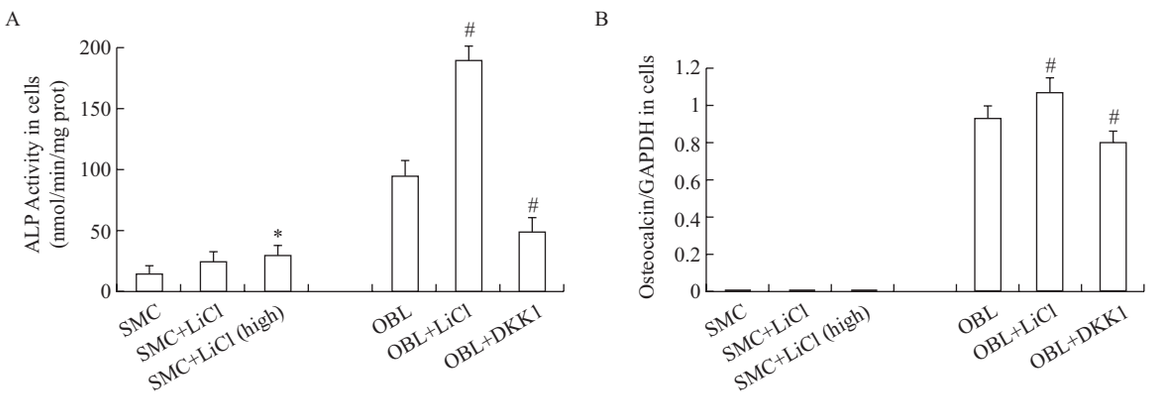


**Fig. 5** The impact of Wnt/β-catenin pathway on the differentiation of OBL cells

A: ALP activity was increased slightly in SMCs treated with LiCl but significantly in OBL cells treated with LiCl. B: The expression of osteocalcin was increased significantly in OBL cells treated with LiCl and suppressed by DKK1. Results were obtained from three independent experiments. $^{*}P<0.05$ *vs.* SMC group, $^{\#}P<0.05$ *vs.* OBL group

calcium deposition observed, even if the dose of LiCl was doubled (fig. 4, fig. 5, table 1).

**Table 1 The assay of calcification level of arteries and OBL cells in two models**

| Models and grouping | Calcium content | |
|---|---|---|
| | Early subgroup | Late subgroup |
| Animal model (mg/g) | | |
| Normal | 0.52±0.04 | 0.53±0.03 |
| Normal + LiCl | 0.53±0.05 | 0.53±0.07 |
| Normal + LiCl (high) | 0.53±0.04 | 0.54±0.05 |
| Calcified | 0.86±0.05$^{*}$ | 1.39±0.04$^{*}$ |
| Calcified + LiCl | 1.23±0.06$^{*\#}$ | 1.63±0.05$^{*\#}$ |
| Cell model (μg/mg cellular protein) | | |
| SMC | 30.11±4.92 | 31.36±4.22 |
| SMC + LiCl | 30.54±5.13 | 31.76±5.52 |
| SMC + LiCl (high) | 31.26±3.76 | 31.97±6.55 |
| OBL | 90.34±7.68$^{*}$ | 120.66±7.63$^{*}$ |
| OBL + LiCl | 121.22±8.45$^{*\#}$ | 142.58±8.97$^{*\#}$ |
| OBL + DKK1 | 64.78±8.72$^{*\#}$ | 104.39±6.96$^{*\#}$ |

$^{*}P<0.05$ *vs.* normal or SMC group, $^{\#}P<0.05$ *vs.* calcified or OBL group. SMC: smooth muscle cell; OBL: osteoblast-like cells

### 2.3 Wnt/β-Catenin Pathway Significantly Affects the RANKL Expression in the Arteries and OBL Cells

After treatment with LiCl, the expression levels of RANKL in arteries and OBL cells were significantly increased ($P<0.05$), but the expression levels of OPG were slightly increased, and the ratio of OPG/RANKL was decreased ($P<0.05$). The effects were just the opposite in the OBL cells when the cells were treated with DKK1, an inhibitor of Wnt/β-catenin pathway ($P<0.05$) (fig. 6, fig. 7).

## 3 DISCUSSION

Arterial calcification is a pathological deposition of calcium and phosphorus on the arterial wall, and is directly associated with increased incidence of many cardiovascular events[25]. In this study, we observed that the expression levels of β-catenin were significantly increased in LiCl-treated calcified arteries and OBL cells, which indicated the activation of Wnt/β-catenin

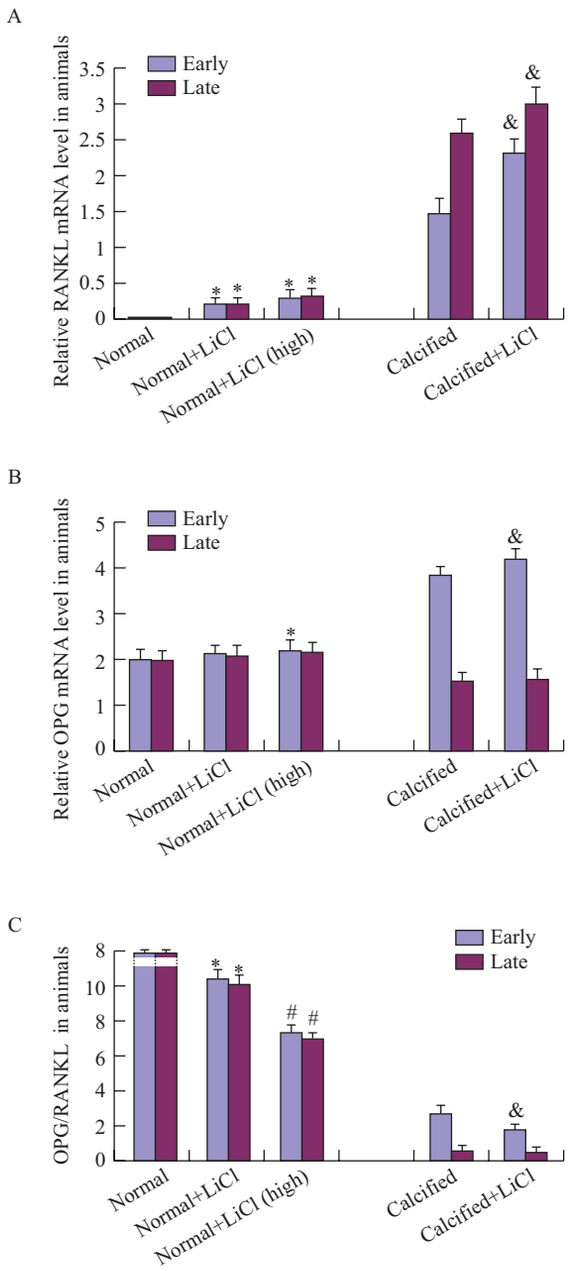


**Fig. 6** The impact of Wnt/β-catenin pathway on the expression of OPG/RANKL in arteries of animals
A: The expression of RANKL was increased significantly in arteries of rats treated with LiCl, especially in the early subgroups; B: The expression of OPG was increased slightly in arteries of rats treated with LiCl; C: the ratio of OPG/RANKL was decreased in arteries of rats treated with LiCl. $^{*}P<0.05$ *vs.* normal group, $^{\#}P<0.05$ *vs.* normal + LiCl group. $^{\&}P<0.05$ *vs.* calcified group

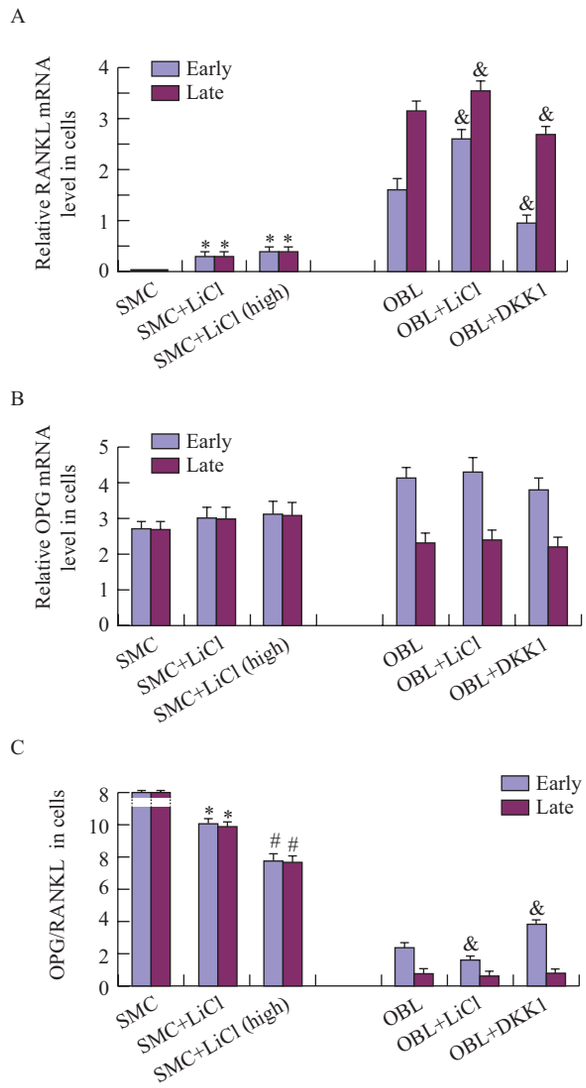


**Fig. 7** The impact of Wnt/β-catenin pathway on OPG/RANKL of OBL cells
A: Real-time PCR showed that the expression of RANKL was increased significantly in SMCs or OBL cells treated with LiCl; B: The expression of OPG was increased slightly in SMCs or OBL cells treated with LiCl; C: The ratio of OPG/RANKL was decreased in SMCs or OBL cells treated with LiCl. The responses were opposite when OBL cells were treated with DKK1. Results were obtained from three independent experiments. $^{*}P<0.05$ *vs.* SMC group, $^{\#}P<0.05$ *vs.* SMC + LiCl group, $^{\&}P<0.05$ *vs.* OBL group. SMC: smooth muscle cell; OBL: osteoblast-like cells

pathway. Additionally, it was found that the activation of Wnt/β-catenin pathway significantly promoted the activity of ALP, a phenotypic marker in the early stage of osteoblast differentiation, and the expression of osteocalcin, a late marker for bone formation, and calcium contents in calcified arteries and OBL cells, and such effects were reversed by treatment with DKK1, an inhibitor of Wnt/β-catenin pathway. Moreover, the expression levels of Wnt3a and Wnt7a proteins, activators of the Wnt/β-catenin pathway, were upregulated in the calcified arteries and OBL cells, which further confirmed the relationship between the Wnt/β-catenin pathway and the calcification. This study also showed that the Wnt/β-catenin pathway had more significant effects on the calcification in the early stage.

Arterial calcification is considered an ectopia ossification process, and some studies demonstrated that the activation of Wnt/β-catenin pathway promoted the ossification also especially in the early stage of the bone formation, and however the mechanisms remain elusive[26, 27]. In the present study, it was revealed that the activation of Wnt/β-catenin pathway increased the calcification development only in the calcified models, but it failed to induce the calcification in the normal models. Several factors have been reported to be responsible for calcification, such as abnormality of the calcium and phosphorus metabolism, the apoptosis of the SMCs, the formation of the matrix vesicle. We assume that without some co-existing factors, the calcification is hard to be formed, even under procalcifying conditions. Therefore, the activation of Wnt/β-catenin pathway alone could not induce the calcification formation in the normal conditions.

In addition, we found that activation of Wnt/β-catenin pathway increased the expression of RANKL more than OPG, resulting in the decreased ratio of OPG/RANKL after LiCl treatment. Moreover, the increased expression of RANKL caused by LiCl in the arteries and OBL cells was more significant in the early subgroups than in the late subgroups. As mentioned above, the activation of Wnt/β-catenin pathway promoted the calcification level more distinctly in the early stages of the calcification, which might be interpreted to be caused by the increased RANKL expression. Many studies have shown that RANKL can promote calcification partly by upregulating the expression of inflammation factors and bone morphogenic proteins (BMPs), and inhibiting the expression of matrix Gla protein (MGP)[28, 29].

In summary, the activity level of Wnt/β-catenin pathway had positive correlation with the level of arterial or cell calcification, activated Wnt/β-catenin pathway during the calcification promoted the calcification level, especially in the early stage, which was probably caused by increasing the expression of RANKL, Wnt3a, and Wnt7a proteins. In the future studies, we will attempt to use the transgenic β-catenin animals, and further examine the exact role of Wnt3a and Wnt7a proteins in order to identify key regulatory points and interactions between Wnt/β-catenin pathway and AMC. The current study confirmed the activation of Wnt/β-catenin pathway promoted the arterial calcification and also indicated that the Wnt/β-catenin pathway could be a new therapy target for the arterial calcification in the future.

**Conflict of Interest Statement**

We declare that we have no conflict of interest.

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