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Nitrogen-containing bisphosphonate for vascular calcification: animal experiments, systematic review and meta-analysis

Wei Xu[1,2,3*], Guoyuan Lu[3], Lifeng Gong[1,2], Weigang Tang[1,2] and Wei Jiang[1,2]

Abstract

Background The purpose of our study was to explore the effect of nitrogen-containing bisphosphonate (N-BP) on vascular calcification (VC) through animal experiments and a meta-analysis.

Methods In our animal experiments, Sprague-Dawley (SD) rats were randomly divided into a control group, a VC group, a low-dose zoledronic acid (ZOL) (20 μg/kg) group and a high-dose ZOL (100 μg/kg) group. The calcification of the aortic arch was observed by alizarin red staining. The calcium content of the aortic arch was measured. In our systematic review and meta-analysis, databases, including PubMed, Embase, the Cochrane Library, China National Knowledge Infrastructure (CNKI), and the Wanfang database, were searched from their inception to December 20, 2023. Eligible studies comparing N-BP versus no N-BP in the treatment of VC were included.

Results In our animal experiment, the red-stained calcification structure in the low-dose ZOL group was slightly reduced and the red-stained calcification structure in the high-dose ZOL group was significantly reduced compared with that in the VC. The calcium content in the low-dose ZOL group was slightly lower than that in the VC group, but the difference was not significant ($P=0.08$). The calcium content in the high-dose ZOL group was significantly lower than that in the VC group ($P<0.0001$). Our meta-analysis of human studies revealed that N-BP did not reduce the arterial calcification score ($P=0.46$). Our meta-analysis of animal studies revealed that N-BP did not significantly reduce the arterial calcification score ($P=0.09$), but N-BP reduced the arterial calcification area ($P<0.00001$), arterial calcium content ($P=0.009$) and PO_4 content ($P=0.0001$).

Conclusions Our animal experiment revealed that high-dose ZOL inhibited VC, but low-dose ZOL did not significantly inhibit VC. Our meta-analysis of human studies revealed that N-BP was not effective in the treatment of VC, but our meta-analysis of animal studies suggested a role of N-BP in inhibiting VC.

Keywords Nitrogen-containing bisphosphonate, Vascular calcification, Animal experiments, Meta-analysis

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Background

Vascular calcification (VC) is defined as the deposition of phosphate-calcium crystals in the cardiovascular system, and it increases the incidence and mortality rate of cardiovascular diseases [1, 2]. At present, there is no effective treatment for VC. Therefore, exploring the mechanism underlying VC and identifying drugs to inhibit VC are very important.

In recent years, epidemiological and clinical studies have shown that patients with low bone density have a significantly increased risk of VC [3, 4]. Some studies have also suggested that drugs that are effective for treating osteoporosis might be effective for treating VC [5, 6]. Bisphosphonate is the first choice for the treatment of osteoporosis [7]; this agent is divided into non-nitrogen-containing bisphosphonate (Non-N-BP) and nitrogen-containing bisphosphonate (N-BP) according to the chemical structure and molecular mechanism. N-BP, such as zoledronic acid (ZOL), is a second or newer generation of bisphosphonate that has a high affinity for bone tissue and can inhibit the activity of farnesyl pyrophosphate synthetase, leading to osteoclast apoptosis [8]. At present, the use of N-BP in the treatment of VC has been studied in humans and animals. However, the efficacy of N-BP in the treatment of VC is uncertain. Therefore, we explored the effect of N-BP on VC through animal experiments. Moreover, we conducted a systematic review and meta-analysis of studies in humans and animals to evaluate the efficacy of N-BPs in the treatment of VC.

Methods

Animal experiments

Experimental protocol

Twenty-four 8-week-old male Sprague-Dawley (SD) rats weighing 250–300 g were purchased from Huachuang Xinuo Pharmaceutical Technology (China). All the rats were randomly divided into four groups, with six rats in each group; the groups included the control group, VC group, low-dose ZOL group and high-dose ZOL group. The VC model was established in the VC group, low-dose ZOL group and high-dose ZOL group. The method for establishing the VC model was as follows [9, 10]. Adenine (450 mg/kg/day) was administered by gavage in the first week, 300 mg/kg/day adenine was administered by gavage in the second to fourth weeks, and high phosphorus feed (1.8% phosphorus, 1% calcium) was provided at the same time. The low-dose and high-dose ZOL groups were given an intraperitoneal injection of 20–100 μg/kg ZOL once a week for 4 weeks. ZOL was administered on the first day of VC modelling. The VC group was given an intraperitoneal injection of the same volume of normal saline. The control group was provided with ordinary feed (0.6% phosphorus, 1% calcium) and administered an equal volume of normal saline by intraperitoneal injection. After 4 weeks, the rats were euthanized by intraperitoneal injection of 150 mg/kg phenobarbital, and the aorta tissues of the rats were extracted. The animal experiments were approved by the Institutional Animal Care Committee at Zhujiang Hospital, Jiangsu University, China (UJS-IACUC-AP-2023030310).

Alizarin red staining

Alizarin red staining was performed to detect calcium deposition. The aortic arch tissues of the rats in each group were embedded in paraffin, sectioned, roasted, dewaxed and dehydrated. The tissue sections were incubated with 1% Alizarin red S solution (Solarbio, China) at room temperature for 1 h and then washed with double steaming water. Then, the tissue sections were sealed, and images were acquired by microscopy.

Determination of the calcium content

The calcium contents were determined with a calcium assay kit (Beyotime, China). The aortic tissues that were collected from each group were placed in a centrifuge tube, to which the sample lysate was added. The samples were then homogenized with a homogenizer and centrifuged at 12,000 rpm at 4 °C for 5 min, and then, the supernatants were collected. The samples were subsequently added to the detection buffer and colour developing solution and incubated at room temperature for 10 min. Finally, the absorbance of the samples at 575 nm was measured with a microplate reader, and the calcium content of the samples was calculated by a standard curve.

Statistical analysis

All the data are presented as the means ± SDs. Differences among the groups were compared using one-way ANOVA. All the statistical analyses were performed with SPSS 20.0 software. Graphs were plotted with GraphPad Prism 8.0 software. $P < 0.05$ was considered statistically significant.

Meta-analysis

Search strategy

Our systematic review and meta-analysis was reported in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines. We searched the PubMed, Embase, Cochrane Library, China National Knowledge Infrastructure (CNKI), and Wanfang databases from their inception to December 20, 2023. The combined text and MeSH terms included ("nitrogen-containing bisphosphonate" or "minodronicacid" or "alendronate" or "risedronate" or "ibandronate" or "zoledronate" or "pamidronate") and ("vascular calcification"). In addition, the relevant references and cited papers were manually searched to

identify additional studies that met the inclusion criteria. There were no language restrictions.

Inclusion and exclusion criteria

The inclusion criteria were the Population, Intervention, Control, and Outcomes (PICO) strategy. The population included patients with VC. The intervention studied involved the use of N-BP. The comparison was no N-BP treatment. The outcomes were the assessment of VC, which included at least one of several indicators, such as the arterial calcification score, arterial calcification area, and arterial calcium or PO_4 contents.

The exclusion criteria were (1) case series, comments, and reviews; (2) no control group; and (3) lack of relevant outcome data.

Data extraction and quality assessment

Data were extracted independently by two investigators using standard data extraction forms. In the case of disagreement, a third investigator was consulted. We extracted data such as the first author, year of publication, location, study design, population, specific methods used in the experimental and control groups, follow-up period, sample size, mean age, sex, weight, and treatment outcomes. The Cochrane assessment tool was used to assess the quality of human RCTs [11], whereas the Newcastle–Ottawa scale (NOS) was used to assess human nonrandomized studies [12]. The Systematic Review Centre for Laboratory Animal Experiments (SYRCLE) tool was used to assess the quality of the animal studies [13].

Statistical analysis

This meta-analysis was performed using Review Manager Version 5.3 (Cochrane Collaboration). We summarized treatment outcomes as weighted mean differences for continuous variables with 95% confidence intervals (CIs). $P<0.05$ was considered statistically significant. We used the I^2 statistic to assess heterogeneity among studies. We considered $I^2>50\%$ and $P<0.10$ to indicate significant heterogeneity. Meta-analysis with insignificant heterogeneity was performed using the fixed-effects model. For meta-analyses with significant heterogeneity, the random-effects model was used. Publication bias was assessed using subgroup analysis or sensitivity analyses.

Results

The results of the animal experiments

Results of alizarin red staining in rats with VC treated with ZOL

As shown in Fig. 1, the red-stained structure represents the calcification in the aortic arch. The control group had no obvious red calcification structure. The VC group had an obvious red calcification structure. Compared with that in the VC group, the red calcification structure in the low-dose ZOL group was slightly reduced, and the red calcification structure in the high-dose ZOL group was significantly reduced.

Results of calcium content determination in rats with VC treated with ZOL

The results of calcium content assessment in the aortic are shown in Fig. 2. The calcium content in the VC group was significantly higher than that in the control group ($P<0.0001$). The calcium content in the low-dose ZOL group was slightly lower than that in the VC group, but the difference was not significant ($P=0.08$). The calcium content in the high-dose ZOL group was significantly lower than that in the VC group ($P<0.0001$).

Results of the meta-analysis

Study selection and characteristics

A flow diagram of the selection process is shown in Fig. 3. Ultimately, a total of eleven studies were included in this meta-analysis [14–24]. Among the eleven studies, three were human studies, and eight were animal studies. The

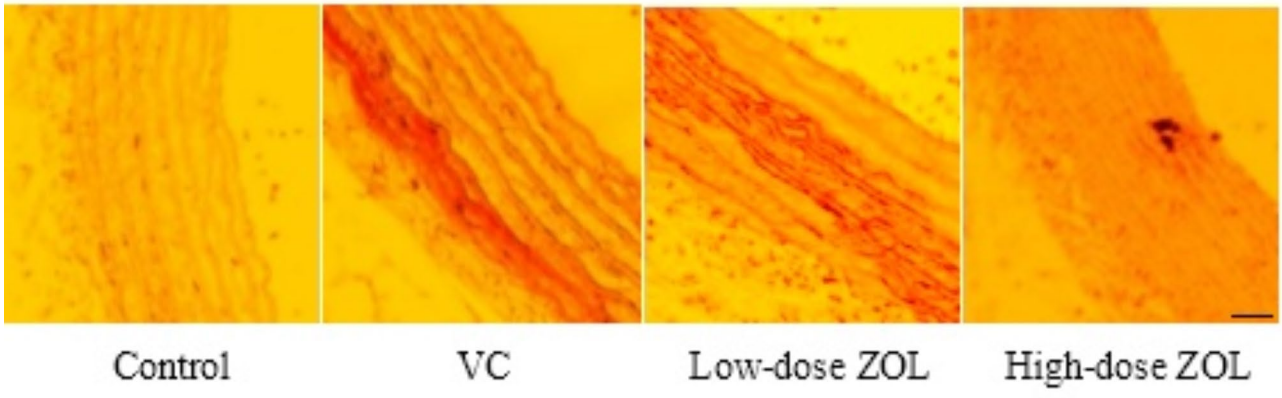

Fig. 1 Alizarin red staining of aortic arch.Scale bar = 50 μm

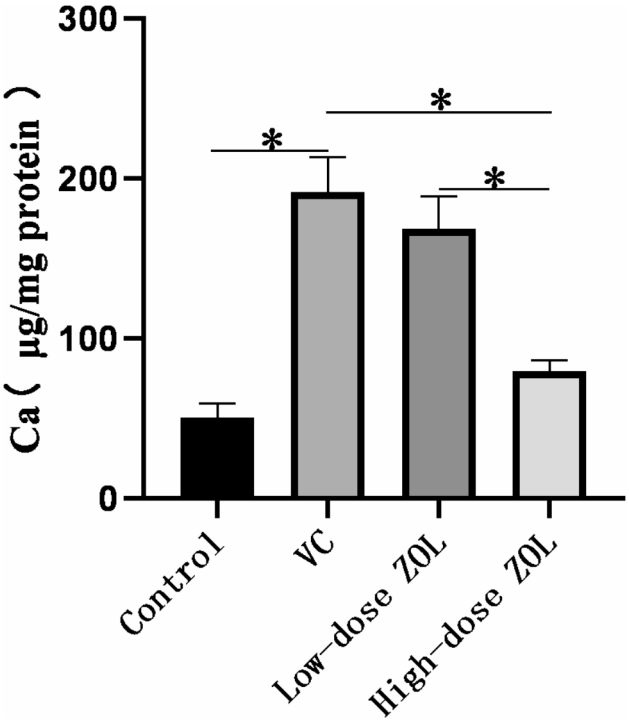

Fig. 2 Calcium content of aortic tissues. * $p < 0.0001$, $n = 6$

risk of bias in the included human RCTs was moderate. The human nonrandomized studies with scores of ≥6 points were considered to be of high quality. The risk of bias in the included animal studies was moderate. The baseline characteristics of the human studies are listed in Table 1, and the baseline characteristics of the animal studies are listed in Table 2. The Cochrane assessments are listed in Table 3, the NOS assessments are listed in Table 4, and the SYRCLE assessments are listed in Table 5.

Results of the arterial calcification score in human and animal studies

Data about the arterial calcification score after N-BP or no N-BP treatment were reported in three human studies and two animal studies. A subgroup analysis was performed according to human and animal studies. In the human subgroup, there was no significant difference between the N-BP and no N-BP treatment groups in terms of the arterial calcification score (SMD −0.11, 95% CI -0.40—0.18, $P = 0.46$). In the animal subgroup, there was no significant difference between the N-BP and no N-BP treatment groups in terms of the arterial calcification score (SMD −1.45, 95% CI -3.10—0.20, $P = 0.09$). When the results of all the subgroups were summarized,

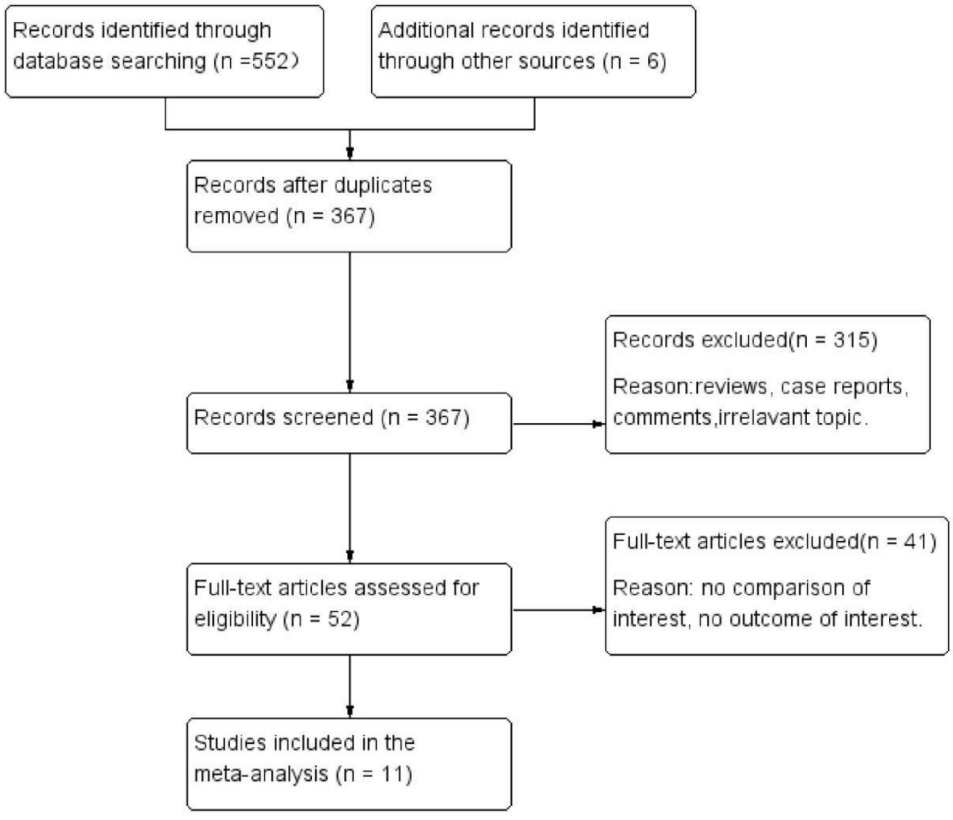

Fig. 3 Flow diagram of the literature search

Table 1 Characteristics of the included human studies

Study (year)	Country	Design	Follow-up period	Population	Treatment group	Comparison group	Sample size (Treatment group/ Comparion group)	Mean age (years)	Male (*n*,%)	Assessment of VC
Hill (2002)	America	Matched-control study	24 months	Patients with osteoporosis	Alendronate sodium 10 mg po daily for a mean of 24 months	No treatment	56 56	-	-	Coronary arterial calcification scores by CT
Torregrosa (2010)	Spain	RCT	12 months	Kidney transplant patients	Risedronate 35 mg po weekly, Calcium and vitamin D	Calcium and vitamin D	52 49	47.4 ± 14.1 50.7 ± 15.5	28(53.8) 27(55.1)	VC score by abdomen and hand X-ray
Okamoto (2014)	Japan	Prospective study	24 months	Kidney Transplant patients	Alendronate 35 mg/week for 24 months	No treatment	5 7	52.8 ± 12.6 52.9 ± 7.3	4(80) 4(57)	Abdominal arterial calcification by CT

there was also no significant difference between the N-BP and no N-BP treatments in terms of the arterial calcification score (SMD − 0.59, 95% CI -1.27—0.10, $P = 0.09$) (Fig. 4A).

Results of the arterial calcification area in animal studies

The data about the arterial calcification area after N-BP or no N-BP treatment were reported in only two animal studies. The arterial calcification area in the N-BP treatment group was significantly lower than that in the no N-BP treatment group (SMD − 2.74, 95% CI -3.48—-2.00, $P < 0.00001$) (Fig. 4B).

Arterial calcium content in animal studies

Data about the arterial calcium content after N-BP or no N-BP treatment were reported in only four animal studies. The arterial calcium content in the N-BP treatment group was significantly lower than that in the no N-BP treatment group (SMD − 4.29, 95% CI -7.51—-1.07, $P = 0.009$) (Fig. 4C).

Results of the arterial PO_4 content in animal studies

Data about the arterial PO_4 content after N-BP or no N-BP treatment were reported in only two animal studies. In the study by Price (2001), the groups treated with alendronate were divided into two groups according to the dose of alendronate used: 0.025 and 0.25 mg/kg/day. The arterial PO_4 content in the N-BP treatment group was significantly lower than that in the no N-BP treatment group (SMD − 2.41, 95% CI -3.65—-1.18, $P = 0.0001$) (Fig. 4D).

Sensitivity analyses

The sensitivity analyses for all the results after N-BP or no N-BP treatment were used to assess the dependability of the results. Regarding the arterial calcification score in the animal subgroup, when we removed the study of Synetos (2018), the arterial calcification score in the N-BP treatment group was lower than that in the no N-BP treatment group ($P < 0.05$). Other results remained unchanged when we removed one study at a time.

Discussion

Our animal experiments revealed that high-dose ZOL inhibited VC, but low-dose ZOL did not significantly inhibit VC. Moreover, our meta-analysis showed that N-BP did not inhibit VC in patients, but N-BP significantly inhibited VC in an animal model of VC.

Recent studies have shown that drugs that are effective for treating osteoporosis might be effective for treating VC [5, 6]. N-BP is a frequently used treatment for osteoporosis. According to our meta-analysis, N-BP is not an effective treatment for VC in humans, but the results of animal studies suggest a role of N-BP in inhibiting VC. In previous studies, N-BP also inhibited the osteogenic differentiation and mineralization of vascular smooth muscle cells, which are the driving steps of VC [25, 26]. The reason why N-BP is not effective against VC in humans is not clear and might be related to the dosage, type, potency and administration route of N-BP [27, 28].

We also conducted animal studies to explore the role of N-BP in inhibiting VC. In our animal experiments, high-dose ZOL inhibited VC, but low-dose ZOL did not significantly inhibit VC. The low and high doses of ZOL administered to the rats were 20 and 100 μg/kg, respectively. The ZOL dose used in our animal experiments was the ZOL dose that is used for the treatment of osteoporosis in humans. The peak serum concentration of ZOL in the human body following a 4-mg dose ranges from 1 to 5 μM [29]. However, low-dose ZOL did not significantly inhibit VC, possibly because the serum concentration of ZOL after low-dose ZOL administration could not be sustained to effectively treat VC or because of the different affinities of ZOL for bone and vascular tissues [27, 28, 30].

There were several limitations in our study. First, there were differences in the dose, type, potency, and administration route of N-BP among the studies that were

Table 2 Characteristics of the included animal studies

Study (year)	Country	Species(Age, Sex, Weight)	The disease of animal model	Treatment group	Comparison group	Sample size (Treatment group/ Comparion group)	Follow-up period	Outcome evaluated
Price(2006)	USA	Rat(13 weeks, 371.4 ± 8.4 g)	Uremia model using 0.75% adenine diet for 4 weeks	2.5% protein diet, Ibandronate ic at a dose of 0.25 mg/kg/day beginning 12 days after the start of the adenine diet	2.5% Protein diet	5 6	4 weeks after the start of the adenine diet	Thoracic aorta calcium and PO_4 determined colorimetrically
Synetos(2018)	Greece	New Zealand rabbits (3 months, male, 3.7 ± 0.2 kg)	Aortic valve stenosis model using vitamin D -enriched atherogenic diet for 3 weeks	Local delivery of a mixture containing 500 μg/l zoledronate on the cusps of the aortic valve using a dedicated balloon catheter	A placebo mixture administered with the same process	8 8	28 days after local drug delivery	Progression of aortic valve calcification by PET/CT Imaging, aortic valve calcification area by von Kossa staining
Synetos(2014)	Greece	New Zealand rabbits(3.8 ± 0.5 kg)	Arterial calcification and atherosclerosis model using vitamin D -enriched atherogenic diet for 3 weeks	Local delivery of a mixture containing 500 μg/l zoledronate on the vascular wall of the iliac artery, using a dedicated balloon catheter	A placebo mixture administered on the contralateral iliac artery with the same process	16	28 days after local drug delivery	The calcium content of iliac artery by computer-assisted histomorphometry
Li Huan(2006)	China	SD Rat (4 weeks, male)	Arterial calcification model using warfarin and vitamin D for 4 days	Alendronate ic at a dose of 1 mg/kg/day starting 4 days before the first warfarin and vitamin D injection	Equal amounts of saline ic	6 6	4 days after the first warfarin and vitamin D injection	Aortic calcification area by von Kossa staining
Price(2001)	USA	Rats(42 days, male)	Arterial calcification model using warfarin for 4 weeks	Alendronate ic at a dose of 0.025 or 0.25 mg/kg/day starting 4 days before the first warfarin injection	No treatment	8 8	4 weeks after the first warfarin injection	Carotid PO_4 determined colorimetrically
Jia peng(2012)	China	SD Rat (5 weeks, male, 150–200 g)	Arterial calcification model using warfarin and vitamin D for 4 days	Alendronate ic at a dose of 1 mg/kg/day starting 4 days before the first warfarin and vitamin D injection	Equal amounts of saline ic	20 20	8 days after the first alendronate injection	Thoracic aorta calcium determined colorimetrically
Yang liyuan (2008)	China	SD Rat(4 weeks, male, 200–250 g)	Arterial calcification model using warfarin and vitamin D for 4 days	Alendronate ic at a dose of 1 mg/kg/day starting 4 days before the first warfarin and vitamin D injection	Equal amounts of saline ic	6 6	8 days after the first alendronate injection	Thoracic aorta calcium determined colorimetrically
Guo liming(2008)	China	SD Rat(female)	Arterial calcification model using subtotal nephrectomy with vitamin D, high phosphorus and calcium diet for 4 weeks	Alendronate ic at a dose of 0.25 mg/kg/day after nephrectomy	No treatment	10 10	4 weeks after nephrectomy	Thoracic aorta calcium by automatic biochemical measurement

Table 3 Quality assessment of randomized control trial

Study	Random sequence generation	Allocation concealment	Blinding of participants and personnel	Incomplete outcome data	Selective reporting	Other bias
Torregrosa (2010)	?	+	?	+	+	?

The randomized control trial was evaluated using the Cochrane assessment tool.+,low risk of bias;?,unclear risk of bias; -,high risk of bias

Table 4 Quality assessment of non-randomized control trial

Studies	Selection	Comparability	Outcome	Score
Hill(2002)	⋆⋆⋆⋆	⋆	⋆⋆	7
Okamoto(2014)	⋆⋆⋆⋆	⋆	⋆⋆	7

The Cohort studies were evaluated using the Newcastle-Ottawa scale, which are comprised of the study of selection (Representativeness of the exposed group, Representativeness of the non exposed group, Ascertainment of exposure, Demonstration that outcome of interest was not present at start of study), group comparability(Controls for the most important factor, Controls for any additional factor), outcome measures (Assessment of outcome, Was follow-up long enough for outcomes to occur, Adequacy of follow up of cohorts), a total of nine points. ⋆, 1 point

Table 5 Quality assessment of animal experiment

Studies	①	②	③	④	⑤	⑥	⑦	⑧	⑨	⑩
Price(2006)	?	?	?	?	?	?	?	+	+	?
Synetos(2018)	+	+	?	+	+	?	+	+	+	?
Synetos(2014)	?	?	?	?	?	?	+	+	+	?
Li Huan(2006)	+	?	?	?	?	?	?	+	+	?
Price(2001)	?	?	?	?	?	?	+	+	+	?
Jia peng(2012)	+	?	?	?	?	?	?	+	+	?
Yang liyuan(2008)	+	?	?	?	?	?	?	+	+	?
Guo liming(2008)	+	?	?	?	?	?	?	+	+	?

The animal experiment was evaluated using the SYRCLE tool.① allocation sequence generated;② baseline similar; ③ allocation concealment ④ random housing animal; ⑤ investigator/caregivers blinded; ⑥ random outcome assessment; ⑦ outcome assessor blinded; ⑧ incomplete outcome adequatedly adressed; ⑨ Selective outcome reporting; ⑩ other risks of bias; +,low risk of bias;?,unclear risk of bias; -,high risk of bias

included in our meta-analysis. Second, the number of studies included in our meta-analysis was still too small. Third, in addition to the human literature we included in our meta-analysis, there are several studies of N-BP in the treatment of VC, such as the studies by Nigel 2020 and Cai 2010 [31, 32]. Nigel and Cai reported that N-BP did not inhibit VC, which was consistent with our results. However, we could not extract the valid data we needed.

Conclusions

Our animal experiments revealed that high-dose ZOL inhibited VC, but low-dose ZOL did not significantly inhibit VC. Our meta-analysis of human studies revealed that N-BP was not effective in the treatment of VC, but our meta-analysis of animal studies suggest a role of N-BP in inhibiting VC. To further confirm this conclusion, additional large human RCTs and animal experiments are necessary.

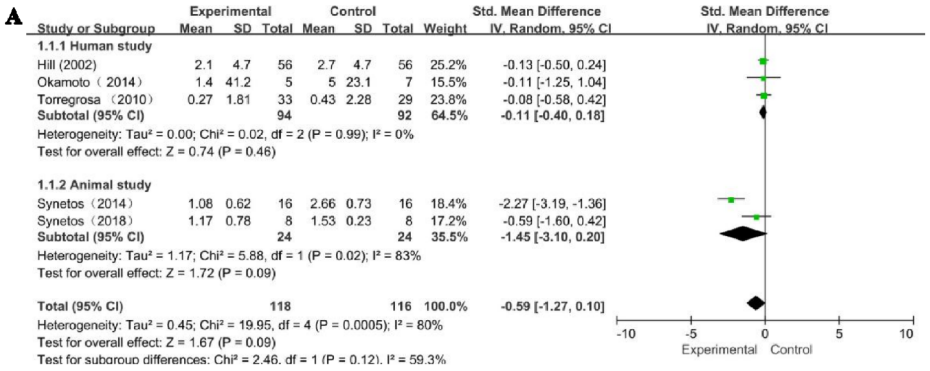

Figure 4. Forest plots comparing the arterial calcification score between N-BP and no N-BP treatment group in human and animal studies.

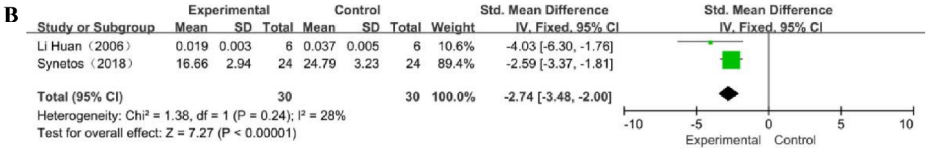

Figure 5. Forest plots comparing the VC area between N-BP and no N-BP treatment group in animal studies.

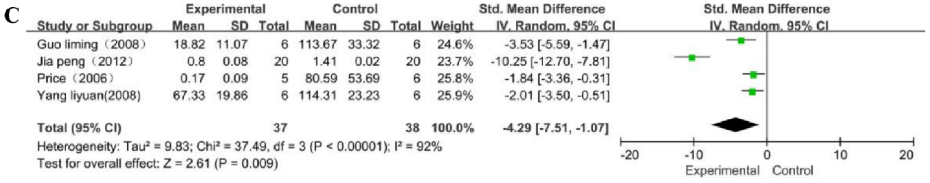

Figure 6. Forest plots comparing the arterial calcium content between N-BP and no N-BP treatment group in animal studies.

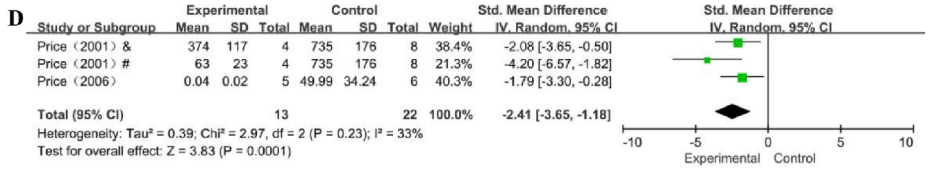

Fig. 4 Forest plots comparing VC index between N-BP and no N-BP treatment group in animal studies. **A**: outcome of arterial calcification score; **B**:outcome of VC area; **C**:outcome of arterial calcium content; **D**:outcome of arterial PO_4 content. & Alendronate at a dose of 0.025 mg/kg/day in Price(2001) study; # Alendronate at a dose of 0.25 mg/kg/day in Price(2001) study

Abbreviations

VC	Vascular calcification
Non-N-BP	Non-nitrogen-containing bisphosphonate
N-BP	Nitrogen-containing bisphosphonate
ZOL	Zoledronic acid
SD	the Sprague-Dawley
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
CNKI	China National Knowledge Infrastructure
PICO	Population, Intervention, Control, and Outcomes
RCTs	Randomized controlled trials
NOS	Newcastle–Ottawa scale
SYRCLE	Systematic Review Centre for Laboratory animal experimentsation
CIs	Confidence intervals

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12872-025-04526-w.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

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Author contributions
Wei Xu and Guoyuan Lu contributed to the conceptualization. Wei Xu, Guoyuan Lu and Lifeng Gong contributed to the animal experiments.Wei Xu, Weigang Tang and Wei Jiang contributed to the meta-analysis. Wei Xu contributed to the analysis of the data and production of figures and tables. Wei Xu and Guoyuan Lu contributed to the writing. All authors approved final manuscript.

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Data availability
Data is provided within the manuscript or supplementary information files.

Declarations

Ethics approval and consent to participate
The animal experiments were approved by the Institutional Animal Care Committee at Zhujiang Hospital, Jiangsu University, China (UJS-IACUC-AP-2023030310). This study is reported in accordance with ARRIVE guidelines.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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