



Investigation of Intravenous Zoledronic Acid Therapy on Circulating Lymphocyte Subpopulation in Patients with Primary Osteoporosis: A Pilot Study

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ABSTRACT

Background: Several studies have investigated the immunomodulating properties of zoledronic acid on T lymphocytes, but the causal relationship between the function of T cells and the efficacy of zoledronic acid has not been elucidated

Objective: To investigate the causal relationship between the function of zoledronic acid and T cells.

Methods: We conducted an observational perspective study to observe the effect of intravenous zoledronic acid once yearly for 2 years on lymphocyte subsets in patients with primary osteoporosis through observing the blood cells analysis and the level of lymphocyte subpopulations before and on day 1, 2, and 3 after first and second administration of intravenous zoledronic acid and bone mineral density 1 year after a single administration of zoledronic acid.

Results: White blood cell count and neutrophils increased, whereas lymphocytes and eosinophils decreased after the first and second zoledronic acid infusion. The count of CD3+T cells, CD3+CD4+T cells, and CD16+CD56+ natural killer lymphocytes decreased from day 1 to day 3 after the first and second zoledronic acid infusion, but the results of second infusion showed no significance.

Conclusions: Further, larger size, more in-depth studies are indicated to examine whether the short-term changes in white blood cells and lymphocyte subtypes noted after 2 once-yearly zoledronic acid injections in this small population of adult patients is associated with the stimulation of immune mechanisms. (Curr Ther Res Clin Exp. 2021; 82:XXX-XXX)

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Introduction

Osteoporosis is characterized by a systemic impairment of bone mass and of the microarchitecture that results in fragility fractures. Over the years, our knowledge about the crosstalk between the bone and immune system has significantly increased; in 2000, the term osteoimmunology was coined. Since then, osteoimmunology, regarding the relationship between the skeletal and immune systems, appears definitely as an interdisciplinary research field that allowed new physiological and pathological interpretations of well-known and common diseases, such as osteoporosis.¹ T lymphocytes

resident in the bone marrow are the key immune cells that regulate bone remodeling and responsiveness of bone cells to parathyroid hormone, in physiological and pathological conditions.²

Bisphosphonates have been used for many years to improve bone mineral density and reduce the risk of fractures in patients with osteoporosis.³ Zoledronic acid inhibits bone resorption by being selectively taken up and absorbed onto mineral surfaces in bone, where it interferes with the action of the bone-resorbing osteoclasts. Zoledronic acid is internalized by osteoclasts and interferes with specific biochemical processes. Several in vitro studies have investigated the immunomodulating properties of zoledronic acid on T lymphocytes,⁴⁻⁸ but the causal relationship between the function of T cells and the efficacy of zoledronic acid has not been elucidated. Based on the immunomodulating role of bis-

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Table 1
Measured parameters at baseline and 1, 2, and 3 days after the first zoledronic acid infusion in patients (n = 30).*

Parameter	Baseline	Day 1	Day 2	Day 3
WBC ($\times 10^9/L$)	6.61 (2.10)	8.55 (2.81) [†]	6.16 (2.06)	5.04 (1.67) [†]
Neutrophils ($\times 10^9/L$)	3.91 (2.24)	6.81 (2.86) [†]	4.57 (1.73)	2.93 (1.41)
Neutrophils (%)	56.88 (12.88)	78.08 (11.08) [†]	73.70 (9.06) [†]	55.5 (13.84)
Monocytes ($\times 10^9/L$)	0.54 (0.29)	0.73 (1.62)	0.43 (0.20)	0.46 (0.20)
Monocytes (%)	8.55 (4.95)	5.50 (2.34)	6.83 (2.37)	9.53 (3.73)
Lymphocytes ($\times 10^9/L$)	1.92 (0.68)	1.15 (0.68) [‡]	1.03 (0.53) [‡]	1.49 (0.62) [†]
Lymphocytes (%)	31.04 (12.69)	14.59 (8.90) [†]	17.21 (7.11) [†]	31.42 (11.65)
Eosinophils ($\times 10^9/L$)	0.15 (0.09)	0.09 (0.11) [‡]	0.09 (0.09) [‡]	0.12 (0.08)
Eosinophils (%)	2.37 (1.48)	1.15 (1.44) [‡]	1.41 (1.32) [‡]	2.40 (1.17)
Basophils ($\times 10^9/L$)	0.04 (0.05)	0.03 (0.02)	0.06 (0.02)	0.02 (0.01)
Basophils (%)	0.51 (0.33)	0.30 (0.19)	0.36 (0.18)	0.47 (0.29)
Ca (mmol/L)	2.29 (0.13)	2.19 (0.13) [†]	2.14 (0.13) [†]	2.14 (0.09) [†]
P (mmol/L)	1.14 (0.17)	1.00 (0.18) [‡]	0.88 (0.17) [‡]	0.90 (0.18) [†]
CD3+ T cells	1210.0 (549.0)	692.5 (615.8) [†]	628.9 (381.3) [†]	1018.9 (448.9)
CD3+ CD4+ T cells	708.1 (286.8)	367.2 (266.1) [†]	366.7 (203.1) [†]	612.1 (270.3)
CD3+ CD8+ T cells	469.5 (311.0)	311.0 (382.0)	246.6 (215.0)	388.8 (308.9)
CD16+ /CD56+ NK cells	348.9 (188.7)	189.5 (122.9) [†]	155.3 (153.7) [†]	254.0 (198.6) [†]
CD19+ B cells	223.9 (126.4)	149.1 (128.5)	140.9 (113.5)	192.2 (160.8)

Ca = calcium; P = phosphorus; NK = natural killer; WBC = white blood cell.

* Values are presented as mean (SD).

[†] $P < 0.01$, compared with baseline.[‡] $P < 0.05$, compared with baseline.

phosphonates and the interaction between bone and the immune system, we conducted an observational perspective study to observe the effect of intravenous zoledronic acid once yearly for 2 years on lymphocyte subsets in patients with primary osteoporosis, through observing the blood cells analysis and the level of lymphocyte subpopulations before and on day 1, 2, and 3 after first and second administration of intravenous zoledronic acid as well as bone turnover markers and bone mineral density 1 year after a single administration of zoledronic acid.

Materials and Methods

Patients were given zoledronic acid (5 mg) once yearly for 2 years via a 60-minute intravenous infusion. Peripheral blood was drawn previous to and 1, 2, and 3 days after zoledronic acid treatment. The primary end point of this study was to determine absolute number and percentage of the following lymphocyte subsets previous to and 1, 2, and 3 days after zoledronic acid treatment: CD3+ T cells, CD4+ T cells, CD8+ T cells; CD19+ B lymphocytes, CD56+ natural killer (NK) lymphocytes. The secondary objective was to evaluate the modification induced by zoledronic acid administration on the bone turnover markers and bone mineral density.

We consecutively enrolled 30 patients with primary osteoporosis followed at the pain department of Jinan Central Hospital. Inclusion criteria included patients who had been diagnosed with primary osteoporosis, no history of administration of aminobisphosphonates, no serious complications such as liver disease, serum calcium and phosphorus at the normal levels, and creatinine clearance >35 mL/min. Exclusion criteria included history of autoimmune or hematological diseases or cancer or acute infections and body temperature $>37.5^\circ\text{C}$. All study participants received 2 annual intravenous infusions of 5 mg zoledronic acid. All participants received daily oral supplements of 600 mg calcium and 400 IU vitamin D3.

Before and 1 day, 2 days, and 3 days after first and second administration of intravenous zoledronic acid, samples of peripheral blood were taken in fasting conditions in the morning. Blood cells analysis was performed using an automated hematology analyzer and plasma electrolytes were measured using an automatic biochemical analyzer (Advia 2120; Siemens, Munich, Germany). Flow cytometry (FACSV antage; B-D, Franklin Lakes, New Jersey)

was used to quantify lymphocyte subsets. Bone mineral density of the lumbar spine was measured using quantitative computed tomography. The bone turnover markers, including levels of serum osteocalcin, serum parathyroid hormone, serum β -isomerized C-terminal crosslinking telopeptide of type I collagen, and serum 25-hydroxyvitamin D were measured using an Elecsys bone marker panel (Roche, Basel, Switzerland) per the manufacturer's instructions.

White blood cell (WBC) and differential cell counts were performed on an automated hematology analyzer (Advia 2120). Anticoagulated peripheral whole blood samples, which were taken using blood collection tubes coated with ethylene-diamine-tetracetic acid, were used to perform the analyses. Lymphocytes were isolated using CD45 versus side scatter (SSC) as a gating strategy. Fifty microliters of blood were distributed into each tube by the automated FACS Sample Prep Assistant II (BD), a mixture of monoclonal antibodies conjugated with different fluorochromes was added, the red blood cells were lysed, and finally the cells were fixed. Lymphocytes were analyzed by flow cytometer with FACS Diva software (BD). Different subsets of T cells were counted using these monoclonal antibodies: APC-conjugated anti-CD3, FITC-conjugated anti-CD4, and PE-Cy7-conjugated anti-CD8. NK cells were counted using APC-CY7-conjugated anti-CD16 and PE-conjugated anti-CD56. B cells were counted using APC-CY7-conjugated anti-CD19. The frequencies of CD3+ T cells, CD3+CD4+ T cells, CD3+ CD8+ T cells, CD19+ B cells, CD16+/CD56+ NK cells were evaluated. The absolute numbers of total lymphocytes were assessed based on the total WBC count and that of the different subsets was determined based on the percentage expression of the cell surface markers. The laboratory used UK NEQAS (www.ukneqas.org.uk) for leukocyte immunophenotyping to ensure external quality.

Data for quantitative variables are described as mean (SD). Peripheral leukocyte and lymphocyte subpopulations were compared in patients before and after zoledronic acid infusion, by the Mann-Whitney U test for nonparametric independent variables. A 2-tailed P value of 0.05 was considered significant. SPSS software version 23.00 (IBM-SPSS Inc, Armonk, NY) was used for statistical analysis.

This study was approved by the ethics committee of Jinan Central Hospital Affiliated to Shandong University, and was performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

Table 2Measured parameters and lymphocyte subsets at baseline and 1, 2, and 3 days after the second zoledronic acid infusion in patients (n = 26).^a

Parameter	Baseline	Day 1	Day 2	Day 3
WBC ($\times 10^9/L$)	6.02 (1.21)	6.95 (2.51)	8.19 (3.04) [†]	6.71 (2.11)
Neutrophils ($\times 10^9/L$)	3.55 (1.11)	4.71 (2.37)	5.93 (2.71) [†]	4.26 (1.92)
Neutrophils (%)	58.02 (10.32)	65.45 (11.24) [†]	71.07 (8.01) [†]	61.2 (11.28)
Monocytes ($\times 10^9/L$)	0.67 (0.43)	0.68 (0.38)	0.76 (0.35)	0.76 (0.42)
Monocytes (%)	12.06 (10.00)	10.52 (7.22)	9.75 (5.09)	12.84 (9.62)
Lymphocytes ($\times 10^9/L$)	1.59 (0.70)	1.38 (0.58)	1.33 (0.60)	1.47 (0.66)
Lymphocytes (%)	26.35 (9.91)	20.83 (9.24)	16.84 (7.48) [†]	22.35 (9.04)
Eosinophils ($\times 10^9/L$)	0.14 (0.08)	0.14 (0.11)	0.12 (0.09)	0.15 (0.08)
Eosinophils (%)	2.53 (1.56)	2.38 (2.08)	1.60 (1.32) [†]	5.52 (1.57)
Basophils ($\times 10^9/L$)	0.03 (0.01)	0.03 (0.01)	0.02 (0.01)	0.03 (0.01)
Basophils (%)	0.51 (0.18)	0.41 (0.24)	0.31 (0.17)	0.43 (0.22)
Ca (mmol/L)	2.2 (0.10)	2.20 (0.12)	2.21 (0.13)	2.20 (0.11)
P (mmol/L)	1.07 (0.17)	1.02 (0.14)	1.01 (0.23)	0.98 (0.17)
CD3+ T cells	1089.2 (494.0)	858.9 (373.4)	842.8 (452.5)	975.6 (442.8)
CD3+ CD4+ T cells	654.3 (304.0)	493.4 (241.7)	477.2 (282.8) [‡]	563.5 (276.9)
CD3+ CD8+ T cells	421.1 (270.7)	343.2 (224.3)	355.3 (245.4)	385.6 (256.3)
CD16+ /CD56+ NK cells	317.0 (253.1)	285.9 (249.4)	301.0 (195.8)	359.8 (234.2)
CD19+ B cells	191.3 (99.9)	165.9 (109.0)	167.6 (121.6)	178.8 (111.0)

Ca = calcium; P = phosphorus; NK = natural killer; WBC = white blood cell.

^a Values are presented as mean (SD).[†] $P < 0.01$, compared with baseline.[‡] $P < 0.05$, compared with baseline.**Table 3**Bone turnover markers and lumbar spine bone mineral density (BMD) at baseline and 1 year following intravenous zoledronic acid (n = 26).^a

Year	n	25(OH)D (ng/mL)	N-MID (ng/mL)	PTH (pg/mL)	β -CTX (ng/mL)	BMD (T value)
0	30	18.21 (9.07)	20.08 (22.48)	38.12 (17.66)	417.05 (207.87)	-3.78 (0.76)
1	26	16.46 (7.19)	18.19 (19.03)	41.08 (18.68)	182.11 (87.19) [†]	-2.83 (0.65) [‡]

25(OH)D = serum 25-hydroxyvitamin D; β -CTX = serum β -isomerized C-terminal crosslinking telopeptide of type I collagen; N-MID = N-terminal middle molecular fragment of osteocalcin; PTH = serum parathyroid hormone.^a Values are presented as mean (SD).[†] $P < 0.05$, compared with year 0.[‡] $P < 0.01$, compared with year 0.

Results

A total of 30 patients were recruited in our study. All patients (9 men and 21 women) completed the first administration of intravenous zoledronic acid. The mean (SD) age was 73.28 (6.44) years. Of these, 26 participants (7 men and 19 women) completed the second administration of intravenous zoledronic acid. The mean (SD) age was 75.21 (5.01) years.

WBC, lymphocyte subpopulations, serum calcium, and phosphate concentrations were measured before and 1, 2, and 3 days following the zoledronic acid infusion. Biochemical findings are shown in Tables 1 and 2. WBC and neutrophils increased, whereas lymphocytes and eosinophils decreased 2 days after the first zoledronic acid infusion. Both proportion and absolute number of circulating neutrophils were significantly increased on day 1 and 2 compared with the basal value, whereas the level of lymphocyte and eosinophils significantly decreased ($P < 0.01$). The absolute number of lymphocytes remained significantly lower on day 3, whereas the level of eosinophils returned to baseline values.

A significant increase in the proportion of circulating neutrophils was observed on day 1 after the second infusion, whereas the other cells showed no difference. Significant increases in the total WBC, neutrophils, and proportion of neutrophils were observed on day 2, whereas the proportion of lymphocyte and eosinophils significantly decreased ($P < 0.01$). The peripheral white cell count showed no significant change on day 3 compared with baseline.

Serum calcium and phosphate concentrations decreased significantly from day 1 to 3 after the first zoledronic acid infusion ($P <$

0.01), whereas there was no significant difference after the second infusion.

We analyzed the changes in the percentages and absolute numbers of the different lymphocyte subsets previous to and 1, 2, and 3 days after both the first and second zoledronic acid treatment. Treatment with a single-dose of zoledronic acid in vivo caused a significant decrease of the count of CD3+ T cells (from 1210/ μ L [549/ μ L] to 692.5/ μ L [615.8/ μ L]), CD3+CD4+ T cells (from 708.1/ μ L [286.8/ μ L] to 367.2/ μ L [266.1/ μ L]), and CD16+CD56+ NK cells (from 348.9/ μ L [188.7/ μ L] to 189.5/ μ L [122.9/ μ L]) on day 1 after the first infusion ($P < 0.05$) (Table 1). There were significant decreases in CD3+T cells (from 1210/ μ L [549/ μ L] to 628.9/ μ L [381.3/ μ L]) and CD3+CD4+ T cells (from 708.1/ μ L [286.8/ μ L] to 366.7/ μ L [203.1/ μ L]) on day 2. On day 3, only the count of CD16+CD56+NK cells showed a significant decrease ($P < 0.05$), the count of CD3+ T cells, CD3+CD4+ T cells were still less than the basal value, but showed no significant change ($P > 0.05$). No significant differences were observed in the other lymphocyte subsets on day 3.

The count of CD3+T cells, CD3+CD4+T cells, and CD16+CD56+ NK cells decreased from day 1 to day 3 after the second zoledronic acid infusion, but showed no significant difference. Only the count of CD3+CD4+ T cells showed a significant decrease on day 2, from 654.3/ μ L [304.0/ μ L] to 477.2/ μ L [282.8/ μ L] ($P < 0.05$). On day 3 the count of CD3+CD4+ T cells was still less than the basal value, but showed no significant change (Fig 1).

No significant differences were observed between values before the first and second zoledronic acid infusion for WBC, lymphocyte subsets, T cells, or serum calcium and phosphate concentrations. Mean (SD) lumbar spine (L1-L4) bone mineral density T score increased from -3.78 (0.76) to -2.83 (0.65) after 1

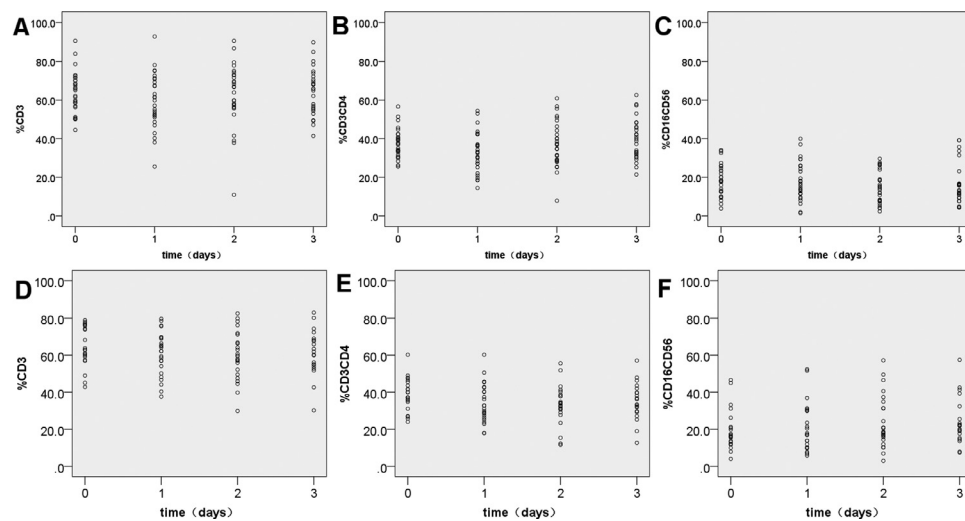


Fig. 1. Proportion of lymphocyte subsets in patients with osteoporosis at baseline and 1, 2 and 3 days after the 1st and 2nd zoledronic acid treatment. (A) (B)(C) There was a significant decrease in the CD3+ and CD3+CD4+ T cell counts on day 2 after the 1st zoledronic acid infusion. On day 3 only the CD16+CD56+NK cell counts showed a significant decrease ($P < 0.05$); the CD3+ and CD3+CD4+ T cell counts were still less than the basal value, but the differences were not statistically significant ($P > 0.05$). (E)(D)(F) The proportion of CD3+, CD3+CD4+ T cells and CD16+CD56+ NK cell counts decreased from day 1 to day 3 after the 2nd zoledronic acid infusion, but showed no significant change. Only the CD3+CD4+ T cell counts showed a significant decrease on day 2 ($P < 0.05$).

year of zoledronic acid treatment. Serum β -isomerized C-terminal crosslinking telopeptide of type I collagen concentrations significantly decreased, whereas the concentrations of serum parathyroid hormone, serum 25-hydroxyvitamin D, and serum osteocalcin showed no significant changes compared with baseline (Table 3).

Discussion

Bisphosphonates are currently considered the mainstay of antiresorptive treatment for osteoporosis and are able to suppress osteoclastogenesis via the inhibition of receptor activator of nuclear factor kappa-B ligand expression on osteoblasts.⁹ Treatment with bisphosphonates, especially when given intravenously, may be associated with increases in body temperature and flu-like symptoms such as malaise, myalgia, and bone pain referred to as an acute phase response. The mechanism has been attributed to release of proinflammatory cytokines, and the mechanism has been further unraveled by showing that it involves release of isopentenylpyrophosphate (IPP) from monocytes and selective receptor mediated activation of $\gamma\delta$ T cells leading to their proliferation and activation.⁷

It has been reported that there was a long-lasting significant decrease not only of $\gamma\delta$ T cells, but also of total WBC, lymphocytes, CD4+ T cells, and eosinophils 1 year after the first infusion of zoledronic acid.¹⁰ We are unaware of any studies examining changes in total WBC, lymphocytes, eosinophils, CD3+T cells, CD3+CD4+ T cells, and CD16+CD56+ NK cells before and on days 1 to 3 after the first and second infusion of zoledronic acid as was done in this pilot study.

We have found that both the proportion and absolute number of circulating neutrophils were significantly increased on day 1 and 2 after the first infusion of zoledronic acid compared with the basal value. However, significant increase of neutrophils, proportion of neutrophils were observed only on day 2 after the second infusion. Studies have revealed that neutrophils play an important role in bone biology, and particularly in the inflammation-induced bone loss.¹¹

Indeed, bone and immune cells are both derived from the bone marrow, and now it has become clear that bone tissue and the immune system often interact. It has been reported that T-cell-

deficient nude mice have normal or elevated bone mineral density.¹² Bisphosphonates, drugs used for some time in the therapy for osteoporosis, are potent inhibitors of osteoclast activity both in the primary and secondary osteoporosis, such as that associated with autoimmune diseases.¹³ Zoledronic acid has been shown to inhibit the mevalonate pathway with a consequent accumulation of isopentenylpyrophosphate (IPP), a phosphoantigen able to induce an expansion and an activation of $\gamma\delta$ T cells.¹⁴

The first zoledronic acid infusion caused a significant decrease in the CD3+T cells and CD3+CD4+ T cell counts on the first day after the infusion. On the second day there were significant decrease for CD3+ T cells and CD3+CD4+ T cells. On day 3, the CD3+ and CD3+CD4+ T cell counts were still less than the basal value but the differences were not statistically significant. The count of CD3+ T cells and CD3+CD4+ T cells decreased from day 1 to day 3 after the second zoledronic acid infusion, but showed no significant difference. Only the CD3+CD4+ T cell counts showed a significant decrease on day 2 after the second infusion.

Osteoclasts share a common origin with T lymphocytes; they both arise from bone marrow hematopoietic stem cells.¹⁵ Activated T lymphocytes are primary sources of receptor activator of nuclear factor kappa-B ligand and tumor necrosis factor alpha responsible for bone destruction observed during various pathological and inflammatory conditions.¹⁶ The final effect of T lymphocytes on bone depends on their activation state and their specific phenotype. Two mechanisms are involved: the binding of T cell costimulatory molecules to their counter receptors on bone cells and their precursors, and the release of cytokines and Wnt ligands that activate Wnt signaling in osteoblastic cell lineage.^{17,18}

The first zoledronic acid infusion caused a significant decrease of the CD16+CD56+ NK cell counts from the first day to the third day after the first infusion. The CD16+CD56+ NK cell counts decreased from day 1 to day 3 after the second zoledronic acid infusion, but the difference was not significant. NK cells have been shown to play a role in the regulation of bone homeostasis. It has been reported that NK cells are able to induce osteoblast cell death.¹⁹ Although our protocol did not include measurement of T helper 17 cells or interleukin 17 concentration in the blood, we believe that the decrease in the CD3+CD4+ T cells and CD16+CD56+ NK lymphocytes found in this study might be—at least partially—explained by the immunomodulating properties of zoledronic acid.

Limitations

Limitations of our study include the small sample size and the fact that Th17 concentrations were not measured. Finally, our data are limited to only 3 days postinjections, that only 1 year of treatment was assessed, and that no evaluation of cytokines or transcription factors was performed.

Conclusions

Further, larger size, more in-depth studies are indicated to examine whether the short-term changes in WBCs and lymphocyte subtypes noted after 2 once-yearly zoledronic acid injections in this small population of adult patients is associated with the stimulation of immune mechanisms.

Declaration of Competing Interest

The authors have indicated that they have no conflicts of interest regarding the content of this article.

Acknowledgments

M. Cui and L. Z. Yu contributed to the conception of the study; M. Cui, N. Zhang, and G. Zhang performed the study; M. Cui, N. Zhang, and L. Han contributed significantly to analysis and manuscript preparation; M. Cui and N. Zhang performed the data analyses and wrote the manuscript; and L. Z. Yu, and G. Zhang helped performed the analysis with constructive discussions.

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