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ORIGINAL ARTICLE

Associations of Changes in Serum Inflammatory Factors, MMP-3, 25(OH)D and Intestinal Flora with Osteoporosis and Disease Activity in Rheumatoid Arthritis Patients

Wang Yong, Li Hongbin, Wang Jing, Zhao Jing, Tie Ning, Bai Lijie

Department of Rheumatology, The Affiliated Hospital of Inner Mongolia Medical University, Hohhot, Inner Mongolia, China

SUMMARY

Background: **To explore the associations of changes in serum inflammatory factors, matrix metalloproteinase-3 (MMP-3), 25-hydroxy vitamin D [25(OH)D], and intestinal flora with osteoporosis and disease activity in rheumatoid arthritis (RA) patients, so as to provide references for clinical diagnosis and treatment.**

Methods: **A total of 98 RA patients were selected as the objects of study (RA group), and divided into active-stage group (n = 56) and remission-stage group (n = 42) according to the disease activity score (DAS28). Another 50 healthy people receiving physical examination in our hospital during the same period were selected as the control group. The changes in serum inflammatory factors, MMP-3, 25(OH)D, and intestinal flora were compared among the three groups, and the osteoporosis of the subjects was analyzed in each group. Moreover, the associations of changes in serum inflammatory factors, MMP-3, 25(OH)D, and intestinal flora with osteoporosis and disease activity in RA patients were analyzed using the Pearson's method.**

Results: **Compared with those in the control group, the levels of serum MMP-3, interleukin-6 (IL-6), IL-10, and C-reactive protein (CRP). The Escherichia coli count were significantly increased, while the level of serum 25(OH)D, bone mineral density (BMD), and Lactobacillus and Bifidobacterium counts were significantly decreased in the active-stage group and remission-stage group, more obviously in active-stage group ($p < 0.05$). The osteoporosis and disease activity in RA patients were positively correlated with serum IL-6, IL-10, CRP, MMP-3, Escherichia coli and BMD, but negatively correlated with 25(OH)D, Lactobacillus and Bifidobacterium ($p < 0.05$), and not correlated with the sharp score ($p > 0.05$).**

Conclusions: **There are certain associations of changes in serum inflammatory factors, MMP-3, 25(OH)D, and intestinal flora with osteoporosis and disease activity in RA patients, showing certain value in clinical application.**
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Correspondence:
Bai Lijie
Department of Rheumatology
The Affiliated Hospital of
Inner Mongolia Medical University
Tongdao North Street No. 1
Huimin District
Hohhot
010050 Inner Mongolia
China
Email: bailijie33@126.com

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KEY WORDS

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INTRODUCTION

Rheumatoid arthritis (RA) is a progressive, erosive, and chronic multisystem inflammatory autoimmune disease, clinically characterized by symmetric erosive arthritis, seriously affecting the quality of life of patients [1]. The pathological characteristics of RA mainly include the neovascularization, constant proliferation of synovial lining cells, infiltration of a large number of inflamma-

tory cells, and invasive, multi-segmental and symmetric inflammatory response in the hands and feet, accompanied by extra-articular organ involvement and excessive expression of serum inflammatory cytokines. Joint deformity and function loss may be caused in severe cases [2].

Alfacalcidol is a medicine for osteoporosis and treat mechanism as follows: (1) promote calcium attraction, (2) improve bone structure, (4) to enhance muscle strength, (5) reduce the pain, (6) promote cartilage repair [3].

Studies have shown that osteoporosis is a common complication of RA patients and an early clinical manifestation of bone defect, which will cause progressive damage to the joint and affect the prognosis of patients with the continuous development of RA [4]. Osteoporosis in RA patients may be related to such factors as disease activity, age, and course of disease [5]. Besides, vitamin D is an important substance for keeping the normal physiological functions of the muscles and bones, and the growth and development of the body. 25-hydroxy vitamin D [25(OH)D] is an important index evaluating the status of vitamin D in the body [6]. There are also studies showing that there is a certain correlation between changes in intestinal flora and RA [7]. Therefore, the associations of changes in serum inflammatory factors, matrix metalloproteinase-3 (MMP-3), 25(OH) D, and intestinal flora with osteoporosis and disease activity in RA patients with alfacalcidol capsules were explored in this paper, so as to provide references for clinical diagnosis and treatment.

MATERIALS AND METHODS

General data

A total of 98 RA patients treated in our hospital from August 2016 to September 2019 were selected as the subjects of study (RA group), and divided into active-stage group (n = 56) and remission-stage group (n = 42) according to the disease activity score (DAS28). After assessment of 28 tender joints (0 - 28), 28 swollen joints (0 - 28), the erythrocyte sedimentation rate, and a patient global assessment (GA) by a Visual Analogue Scale (0 - 100), the DAS28 is calculated as follows: $DAS28 = 0.56\sqrt{t28} + 0.28\sqrt{sw28} + 0.7\ln(ESR) + 0.014GA$ (t-tender joints, sw-swollen joints). The DAS-28 activity evaluation: remission DAS28 < 2.6, low activity DAS28 > 2.6 < 3.2, medium activity DAS28 > 3.2 < 5.1, high activity DAS28 ≥ 5.1. Another 50 healthy people receiving physical examination in our hospital during the same period were selected as the control group. The control group included 16 males and 34 females aged 25 - 53 years old with an average of 38.15 ± 3.16 years old. In the RA group, there were 35 males and 63 females aged 25 - 55 years old with an average of 38.36 ± 3.03 years, and the course of disease was 2.61 ± 0.56 years. The general data such as age and gender showed no statistically significant differences between the two groups, and they were comparable ($p > 0.05$).

Inclusion and exclusion criteria

Inclusion criteria: 1) patients in the RA group met the diagnostic criteria for RA in the Guidelines for the Diagnosis and Treatment of Rheumatoid Arthritis, 2) subjects had complete clinical data, and 3) subjects and/or families agreed and signed the informed consent. Exclusion criteria: 1) subjects complicated with severe dysfunction in the heart, liver or kidney, 2) those complicated with diabetes or malignant tumors, 3) those who were bedridden for a long time or could not be exposed to direct sunlight, 4) those complicated with acute infection or tuberculosis infection, 5) those complicated with parathyroid or thyroid diseases, 6) those who took estrogen/androgen, immunosuppressors, anticoagulants or drugs affecting bone metabolism for a long time, 7) those with blood system diseases, or 8) those who had poor compliance or quit halfway.

Methods

The level of serum 25(OH)D was determined in the RA group, and 25(OH)D < 20 ng/mL indicated vitamin D deficiency. In addition to standard treatment, the RA group took alfacalcidol capsules (NMPN 20041837, CONSUN, 0.25 mg) for 6 months (2 capsules/per day).

Observation indexes

1) Serum inflammatory factors: 5 mL of elbow venous blood was drawn from subjects and centrifuged. The serum separated was stored in a refrigerator at -75°C for later use. The levels of interleukin-6 (IL-6) and IL-10 were measured via enzyme-linked immunosorbent assay (ELISA) using kits (Shanghai Yubo Biotechnology Co., Ltd) strictly according to the instructions. The level of serum C-reactive protein (CRP) was determined via immunoturbidimetry. 2) The levels of serum MMP-3 and 25(OH)D were measured via ELISA using the kits (Addis, UK) strictly according to the instructions. 3) Intestinal flora: Feces specimens were collected from all subjects, diluted with 9 mL of Ringer diluent containing 0.1 g of cysteine, and prepared into suspension at four concentrations (10^{-8} g/L, 10^{-7} g/L, 10^{-6} g/L, and 10^{-5} g/L). Then 100 μg of suspension was streaked onto Lactobacillus medium, Bifidobacterium medium and Mac Conkey medium, and cultured at 37°C for about 24 hours. After anaerobic culture, the colony morphology of Lactobacillus and Bifidobacterium was observed, and the colony-forming units were counted according to the method described by Du XD et al. 4) DAS28: Based on the relevant regulation in the disease activity scoring system, DAS28 > 3.2 indicates the active stage, while DAS28 < 2.6 indicates the remission stage. 5) Bone mineral density (BMD expressed in gm/cm^2 (DXA)): BMD of the right distal radius was measured using a BMD instrument (Guangzhou Nanxiunan Trading Co., Ltd., UNIGAMMA X-RAY PLUS). The decline in the peak BMD by > 2.5 standard deviations compared with

Table 1. Comparison of levels of serum inflammatory factors among the three groups.

Group	IL-6 (ng/L)	IL-10 (ng/L)	CRP (mg/mL)
Control group (n = 50)	19.21 ± 3.21	53.06 ± 8.74	1.02 ± 0.15
Remission-stage group (n = 42)	42.69 ± 5.49	326.87 ± 21.56	3.06 ± 0.59
Active-stage group (n = 56)	302.59 ± 9.69	625.48 ± 33.58	13.05 ± 1.21
F	18.028	9.619	13.351
p	< 0.001	< 0.001	< 0.001

healthy adults of the same race and gender indicates osteoporosis. 6) Sharp score: According to the X-ray stage of both hands, the bone erosion score (0 - 5 points) was given to 17 regions in each wrist, and the joint space narrowing score (0 - 4 points) was given to 18 regions. The Sharp score is the sum of the scores of all items.

Statistical analysis

SPSS19.0 software (IBM SPSS Statistics for Windows; IBM Corp, Armonk, NY, USA) was used for data processing. Measurement data were expressed as mean ± standard deviation ($\overline{x} \pm s$), and *t*-test or F test was performed. Enumeration data were expressed as [n (%)], and chi-square test was performed. The associations of changes in serum inflammatory factors, MMP-3, 25(OH)D, and intestinal flora with osteoporosis and disease activity in RA patients were analyzed using Pearson's method. $p < 0.05$ suggested that the difference was statistically significant.

RESULTS

Comparison of levels of serum inflammatory factors among the three groups

The levels of serum IL-6, IL-10, and CRP were significantly higher in the active-stage group and the remission-stage group than those in the control group, and they rose more significantly in the active-stage group ($p < 0.05$) (Table 1).

Comparison of levels of serum MMP-3 and 25(OH)D among the three groups

The level of serum MMP-3 was obviously higher, while the level of serum 25(OH)D was obviously lower in the active-stage group and the remission-stage group than those in the control group. They were changed more obviously in the active-stage group ($p < 0.05$) (Table 2).

Comparison of changes in intestinal flora among the three groups

The active-stage and remission-stage groups had an evidently increased Escherichia coli count and evidently deceased Lactobacillus and Bifidobacterium counts compared with the control group. The above indexes were changed more evidently in the active-stage group ($p < 0.05$) (Table 3).

Comparison of incidence of osteoporosis among the three groups

Compared with the control group, the incidence rate of osteoporosis was increased in the active-stage and remission-stage groups, more significantly in the active-stage group. The difference was statistically significant ($p < 0.05$) (Table 4).

Changes in BMD and Sharp score in the three groups

Compared with the control group, BMD remarkably declined in the active-stage and remission-stage groups, more remarkably in the active-stage group. The differences were statistically significant ($p < 0.05$) (Table 5).

Correlation analysis of changes in serum inflammatory factors, MMP-3, 25(OH)D and intestinal flora with osteoporosis in RA patients

The osteoporosis in RA patients was positively correlated with serum IL-6, IL-10, CRP, MMP-3, Escherichia coli and BMD, but negatively correlated with 25(OH)D, Lactobacillus and Bifidobacterium ($p < 0.05$), and not correlated with the Sharp score ($p > 0.05$) (Table 6).

Correlation analysis of changes in serum inflammatory factors, MMP-3, 25(OH)D, and intestinal flora with disease activity in RA patients

The disease activity in RA patients was positively correlated with serum IL-6, IL-10, CRP, MMP-3, Escherichia coli and BMD, but negatively correlated with 25(OH)D, Lactobacillus and Bifidobacterium ($p < 0.05$), and not correlated with the Sharp score ($p > 0.05$) (Table 7).

DISCUSSION

RA is a systemic inflammatory autoimmune disease mainly manifested as bone erosion and periostitis, which is characterized by recurrent and continuous onset and a high disability rate [8]. Currently, there has been no authoritative explanation of the pathogenesis of RA in clinic, and it is mostly believed that its pathogen-

Table 2. Comparison of levels of serum MMP-3 and 25(OH)D among the three groups.

Group	MMP-3	25(OH)D (ng/mL)
Control group (n = 50)	8.63 ± 1.03	25.41 ± 3.65
Remission-stage group (n = 42)	12.85 ± 1.21	16.58 ± 3.25
Active-stage group (n = 56)	14.12 ± 1.30	12.51 ± 2.98
F	14.779	20.478
p	< 0.001	< 0.001

Table 3. Comparison of changes in intestinal flora among the three groups (lgCFU/g of feces).

Group	Escherichia coli	Lactobacillus	Bifidobacterium
Control group (n = 50)	3.89 ± 0.36	6.21 ± 0.58	6.33 ± 0.63
Remission-stage group (n = 42)	4.52 ± 0.39	5.13 ± 0.33	4.98 ± 0.59
Active-stage group (n = 56)	5.89 ± 0.28	4.29 ± 0.41	4.22 ± 0.37
F	20.178	11.286	7.717
p	< 0.001	< 0.001	< 0.001

Table 4. Comparison of incidence of osteoporosis among the three groups.

Group	Osteoporosis [n (%)]
Control group (n = 50)	7 (14.00)
Remission-stage group (n = 42)	14 (33.33)
Active-stage group (n = 56)	24 (42.86)
F	3.079
p	0.002

Table 5. Changes in BMD and Sharp score in the three groups.

Group	BMD (gm/cm^2)	Sharp score (point)
Control group (n = 50)	0.83 ± 0.09	16.58 ± 2.15
Remission-stage group (n = 42)	0.65 ± 0.12	16.25 ± 2.22
Active-stage group (n = 56)	0.48 ± 0.08	16.20 ± 2.25
F	26.510	0.500
p	< 0.001	0.310

esis is closely related to the body's inflammatory response and immune dysfunction [9]. Osteoporosis is a common clinical complication of RA, and its pathological basis is bone loss caused by the destruction of bone metabolism. Vitamin D plays an important role in bone calcification [10]. As a sterol derivative able to bind to specific receptors in the body, vitamin D can regulate bone calcium-phosphorus metabolism and also inhibit synthesis and secretion of inflammatory factors through lymphocytes and dendritic cells, thereby participating in the regulation of immune function [11]. DSA28, integrating the laboratory examination indexes, patient's subjective feelings about pain, and objective physical examination, can accurately evaluate the disease activity of RA patients [12]. As important laboratory examination indexes, inflammatory factors can well promote

Table 6. Correlation between relevant indexes and osteoporosis in RA patients.

Item	*r*	p
IL-6	0.393	< 0.001
IL-10	0.422	< 0.001
CRP	0.849	< 0.001
MMP-3	0.569	< 0.001
25(OH)D	-0.429	< 0.000
Escherichia coli	0.183	0.012
Lactobacillus	-0.319	< 0.001
Bifidobacterium	-0.526	< 0.001
BMD (gm/cm^2)	0.698	< 0.001
Sharp score	0.032	0.587

Table 7. Correlation between relevant indexes and disease activity in RA patients.

Item	*r*	p
IL-6	0.849	< 0.001
IL-10	0.698	< 0.001
CRP	0.183	0.012
MMP-3	0.756	< 0.001
25(OH)D	-0.653	< 0.001
Escherichia coli	0.480	< 0.001
Lactobacillus	-0.756	< 0.001
Bifidobacterium	-0.569	< 0.001
BMD (gm/cm^2)	0.755	< 0.001
Sharp score	0.021	0.805

the release of a variety of enzymes and cells, and play a critical role in the occurrence and development of RA [13].

In patients with RA in the active stage, T lymphocytes can activate a large number of cytokines, while vitamin D can inhibit the secretion of inflammatory factors. 25(OH)D is an endogenous immunomodulator that can well inhibit synovial joint damage in RA [14]. Macrophages play a key role in the occurrence of synovitis, and they can ultimately lead to bone destruction. Moreover, estrogen can greatly promote the activation of macrophages, thus exacerbating arthritis. In contrast, vitamin D can suppress the production of corresponding cytokines by macrophages, thus resisting bone destruction [15]. The above findings demonstrate that the disease activity and osteoporosis in RA patients have a negative correlation with the serum 25(OH)D level, consistent with the research results of Skacelova M et al. [16]. ILs play prominent roles in the activation of immune cells and inflammatory response in the body. Sauer BC et al. also found that the pathogenesis of RA may be related to the activation of B cells and relatively increased secretion of antibodies [17]. Meanwhile, due to the specific binding between antibodies and autoantigens, a large number of immune complexes deposit on vascular walls, bone joints and synovium, leading to tissue damage, synovitis, and cartilage destruction. The results in this study suggest that the increased levels of serum inflammatory factors can promote the occurrence of osteoporosis in RA patients, consistent with the research results of Bermas BL et al. [18]. Under the balance between host and flora, the immune cells in intestinal mucosa will interact with microorganisms to jointly promote the maturation of the immune system, thereby effectively avoiding the colonization of harmful bacteria [19]. Intact epithelial cells can well protect the intestinal tract from damage, and few exogenous antigens can pass through them to reach the immune cell storage layer and lamina propria. In the case of leakage of epithelial cells, the permeability of the intestinal wall for micro-

organisms will increase, thus enhancing the inflammatory response, promoting the loss of immunologic tolerance to inherent bacteria, and making antigens and immune cells accumulate in the joints. Finally, the result is RA [20]. In this paper, Escherichia coli (considered a non-probiotic in most clinical studies), Lactobacillus and Bifidobacterium (considered probiotics) were selected for research, and it was found that the intestinal flora was certainly closely related to the occurrence of RA.

In conclusion, there are certain associations of changes in serum inflammatory factors, MMP-3, 25(OH)D, and intestinal flora with osteoporosis and disease activity in RA patients, showing certain value in clinical application.

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Declaration of Interest:

The authors have no conflicts of interest regarding the publication of this paper.

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