Effects and safety of ^{99}Tc-MDP in patients with refractory ankylosing spondylitis: a 2-stage (30-week follow-up) clinical trial

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Abstract

Objective

To evaluate the clinical efficacy and safety in patients with refractory ankylosing spondylitis (AS) initiating ^{99}Tc-MDP therapy and explore the mechanisms.

Methods

Refractory AS patients were enrolled in the clinical trial and received ^{99}Tc-MDP treatments for 3 or 5 courses according to ASAS improvement. Efficacy and safety evaluations were conducted during the follow-up. 37 cytokines were quantified by Luminex at baseline and week 30. p-values<0.05 were considered statistically significant.

Results

51 refractory AS patients were included, with 20 healthy people serving as the control group. The patients were in an active disease state (mean (SD) ASDAS 3.66 (0.83), BASDAI 4.53 (1.92)), 42(82.35%) patients had syndesmophytes. Their cytokines were significantly higher than that in the control group. After 3 courses of ^{99}Tc-MDP treatment, 32 (62.75%) patients achieved ASAS20 improvement, 24 (47.06%) patients achieved a clinically significant improvement (ΔASDAS-CRP≥1.1). 27 patients entered the second stage to complete 5 courses of the treatment, all of whom achieved ASAS20 improvement, 18 (66.67%) patients achieved a clinically significant improvement. All clinical parameters including ASAS and ASDAS significantly improved as the treatment was continued. Cytokines also had significant down-regulation after the treatment, and the reductions had positive correlations with the improvements of disease activity. No serious adverse event was observed.

Conclusion

This investigation confirmed the remarkable efficacy of ^{99}Tc-MDP in a large number of refractory AS patients, and highlighted the mechanism by dramatic regulation on cytokines. ^{99}Tc-MDP was safe in clinical application.

Key words

^{99}Tc-MDP, refractory ankylosing spondylitis, clinical trial

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Introduction

Ankylosing spondylitis (AS) mainly affects the axial spinal column, causing syndesmophytes (enthesiophytes) and ankylosis in advanced disease (1, 2). The pathogenesis of AS still remains unclear; inheritance, infection, circumstance and immunity are all contributing factors (3-5). Recurrent inflammation and unbalanced bone turnover play vital roles in the the progression of AS. Fluctuating inflammation results in pain and swollen, while pathological bone turnover consisted of excessive new bone formation and bone destruction usually cause ankylosis or osteoporosis (6-8).

This study concentrated on refractory AS patients whose disease was considered to be severe and intractable with conventional therapy (9), which means that after 3 to 6 months of medication**,** these patients were still in high disease activity [Ankylosing Spondylitis Disease Activity Score (ASDAS) score ≥1.3 and/or Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) score≥4], and most of the refractory patients were in the advanced stage of the disease.

Technetium-99 conjugated with methylene diphosphonate (^{99}Tc-MDP), also known as Yunke injection, consists of Technetium-99 conjugated with methylene diphosphonate. It has been widely used in the treatment of rheumatoid arthritis (RA) (10). In recent years, we found that AS patients could get symptom alleviation after a short ^{99}Tc-MDP application, but there were few previous studies which focused on small samples and limited numbers of evaluating parameters after short-term ^{99}Tc-MDP use. Moreover, there is a lack of prospective clinical trials referring to systemic and overall evaluation of the clinical response (including ASAS20/40 and ASDAS improvement), as well as inflammatory cytokine response in refractory AS patients initiating long-term therapy of ^{99}Tc-MDP. The underlying mechanisms of ^{99}Tc-MDP in the treatment of AS has not been clarified either.

The present prospective intervention cohort study was the first clinical trial designed to evaluate the efficacy of ^{99}Tc-MDP in patients with refractory AS, and to investigate the underlying mechanisms of ^{99}Tc-MDP in treatment, with emphasis on the regulation of biochemical markers of inflammation. The study also paid attention to the safety of ^{99}Tc-MDP treatment.

Patients and methods

Patients

Patients (18~65 years old) fulfilling the 1984 modified AS New York criteria with the presence of active disease, as defined by ASDAS score ≥1.3 and/or BASDAI score ≥4, were recruited in this investigation. Other criteria of inclusion included a stable dose of non-steroidal anti-inflammatory drugs (NSAIDs) or disease-modifying antirheumatic drugs (DMARDs) throughout the study, and an interruption of biological agents for at least 3 months. The exclusion criteria included allergy and severe complications. The patients were followed up for certain times at regular intervals according to the study procedure. Finally, 51 refractory AS patients with a mean age of 35.84±9.80 years were screened to enter the investigation. Twenty healthy volunteers were enrolled as a control group, with a mean age of 33.35±9.80 years.

Full radiographs of the spine were not the necessary inclusion criteria, but were recommended at baseline. Fifty-one patients had undergone x-ray of the sacroiliac and hip joint, cervical, thoracic and lumbar vertebra, and these radiological data were read by two radiologists and one rheumatologist independently. Bone mineral density (BMD) was tested by DXA in patients, osteopenia (-2.5<T<-1.0) and osteoporosis (T≤-2.5) were defined by WHO criteria (11). The investigation was approved by the medical ethics committee of the Third Affiliated Hospital of SYSU (Guangzhou, China), and informed consents were obtained from all subjects.

Drug administration and study design

The ^{99}Tc-MDP injection used in the present study was obtained from Yunke Pharmaceutical (Chengdu, China). ^{99}Tc-MDP was injected intravenously at a dose of 22 mg/d in 250 ml 0.9% NaCl. One course of ^{99}Tc-MDP treatment was one dose a day for 14 days.

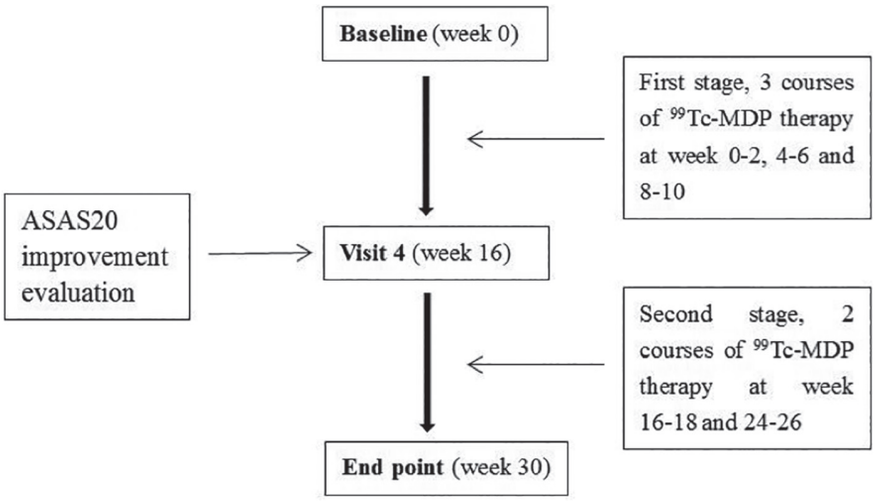

Fig. 1. The two-stage study procedure. Evaluation was performed after the first stage, patients who got ASAS20 improvement would enter the second stage to finish the study.

The prospective study consisted of two stages (Fig. 1). At the first stage, patients received ^{99}Tc-MDP treatment for 3 courses respectively at week 0–2, 4–6 and 8–10. ASAS improvement was evaluated at visit 4 (week 16), patients who had an ASAS20 improvement would enter the second stage to receive the additional 2 courses of injection at week 16–18 and 24–26. Week 30 was the final evaluation point of the follow-up.

Evaluation of efficacy

Clinical evaluation (Table I) was performed six times during the visit (12, 13). The primary clinical parameters for efficacy were the Assessment in SpondyloArthritis International Society (ASAS) and the Ankylosing Spondylitis Disease Activity Score (ASDAS) (14, 15). The ASAS improvement criteria included ASAS20, ASAS40, ASAS 5/6, ASAS partial remission (12, 16, 17). ASDAS (ASDAS-CRP/ASDAS-ESR) was calculated by particular formulae, four disease activity states (inactive disease, moderate, high and very high disease activity) were defined by three ASDAS cut-offs: 1.3, 2.1 and 3.5 units. Improvement responses were defined as: change ≥1.1 units for clinically important improvement and change ≥2.0 units for major improvement (18). Secondary clinical parameters including Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) (19), Bath Ankylosing Spondylitis Disease Functional Index (BASFI) (20), visual analogue scale (VAS) of patient global assessment of disease activity (PGA) and last week spine pain at night/overall due to AS, Bath Ankylosing Spondylitis Metrology Index (BASMI) (21), C-reaction protein (CRP), erythrocyte sedimentation rate (ESR), morning stiffness time and visual analogue scale (VAS) of peripheral joints pain.

Reagents and laboratory test

The serum samples were centrifuged and stored at -80°C until analysed simultaneously. 37 cytokines from the TNF superfamily proteins, IFN family proteins, Treg cytokines, and MMPs (APRIL/TNFSF13, BAFF/TNFSF13B, sCD30/TNFRSF8, sCD163, Chitinase-3-like 1, gp130/sIL-6Rβ, IFN-α2, IFN-β, IFN-γ, IL-2, sIL-6Rα, IL-8, IL-10, IL-11, IL-12 (p40), IL-12 (p70), IL-19, IL-20, IL-22, IL-26, IL-27 (p28), IL-28A/IFN-λ2, IL-29/IFN-λ1, IL-32, IL-34, IL-35, LIGHT/TNFSF14, MMP-1, MMP-2, MMP-3, osteocalcin, osteopotinin, pentraxin-3, sTNF-R1, sTNF-R2, TSLP, TWEAK/TNFSF12) in serum samples were quantified by Luminex. The reagents were obtained from Bio-Rad (California, USA). Twenty patients who fulfilled the investigation were selected to measure the cytokines in serum at baseline (week 0) and at the end point (week 30), which were the critically important moments of the prospective study. The characteristics of the 20 selected patients, with a mean age of 34.90±9.03 years, did not differ from the sample.

Evaluation of safety

Patients were monitored by vital signs (temperature, pulse and blood pressure). Adverse reactions were recorded. Routine blood and urine tests, as well as tests of liver and kidney functions were all performed at each visit.

Statistical analysis

Statistical analysis was performed with SPSS 20.0 software for Windows (SPSS, Chicago, IL, USA). The variables were expressed by mean with standard deviation ($\bar{X}$±s) or median with quartiles (median (Q_1-Q_3)) according to Shapiro-Wilk normality

Table I. Brief scheme of efficacy evaluation.

	Assessment				
Primary parameters					
ASAS improvement criteria	ASAS20 improvement		ASAS40 improvement	ASAS5/6 improvement	ASAS partial remission
ASDAS disease activity states	inactive disease (<1.3)		moderate disease (<2.1)	high disease (≤3.5)	very high disease
ΔASDAS improvement responses	clinically important improvement (≥1.1)		major improvement (≥2.0)		
Secondary parameters					
	BASDAI	BASFI	PGA	spine pain at night	spine pain overall
	BASMI	CRP	ESR	morning stiffness time	peripheral joints pain

test. Paired-sample *t*-test and Wilcoxon signed rank test were used for comparison between paired samples, independent-sample *t*-test and Mann-Whitney U-test were used for comparison between two independent groups, while one-way ANOVA and Kruskal-Wallis H test were used in multi-group comparison of continuous variables. The correlations were presented using Pearson's correlation coefficients or Spearman's correlation coefficients. A two-tailed *p*-value <0.05 was considered significant.

Results

Fifty-one refractory AS patients with a mean age of 35.84±9.80 years were screened to enter the investigation, 20 healthy subjects with a mean age of 33.35±9.80 years served as the control group. 32 out of 51 patients entered the second stage according to ASAS20 improvement after week 16, and 27 patients with a mean age of 37.04±9.41 years completed the second stage and reached the end point.

Baseline outcomes

Table II shows the baseline characteristics of the individuals.

At baseline, ASDAS showed that all of the patients were in a state of active disease (Fig. 2), and 97% of them had high disease activity. Correlation test showed that ASDAS, CRP and ESR were higher in younger patients; CRP and ESR were higher in patients with shorter symptom duration, while BASMI was higher in patients with longer symptom duration. Positive linear correlations between the clinical parameters (except for BASMI) showed the consistency of these parameters in evaluation.

Radiographic examinations of the spine and pelvis were performed at baseline, 42 (82.35%) patients had more than one syndesmophyte, and each patient had 13.61 on average. Lumbar vertebra was most easily affected. The number of syndesmophytes had positive correlations with age and symptom duration, but negative correlations with ASDAS-CRP, ASDAS-ESR, CRP, ESR and morning stiffness time. 98.04% of the patients had grade III or IV sacroiliitis. Thirty-two (82.05%) patients were in a state of osteopenia or osteoporosis.

Table II. Baseline demographic, clinical and radiographic characteristics.

Assessment	AS patients (n=51)	Healthy volunteers (n=20)
Age (years)	35.84 ± 9.80	33.35 ± 9.80
Male gender (%)	94.11	94.70
HLA-B27positive (%)	93.75 (45/48)	
Peripheral arthritis (%)	52.94	
Family history (%)	39.21	
Symptom duration (months)	162.59 ± 104.28	
BMI	21.38 ± 3.77	
NSAIDs (%)	100	
DMARDs (%)	21.56	
Biological agents used (%)	29.41	
Hip arthroplasty (%)	5.88	
Smoking (%)	27.50	
Hepatitis B (%)	27.45	
Lumbar vertebra T score	-1.48 ± 1.31	
Femoral neck T score	-1.59 ± 0.95	
Hip joint T score	-1.38 ± 0.86	

BMI: body mass index; NSAIDs: non-steroidal anti-inflammatory drugs; DMARDs: disease-modifying anti-rheumatic drugs.

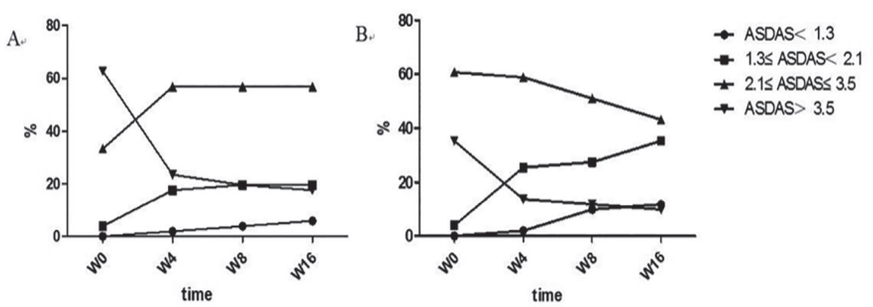

Fig. 2. Longitudinal relationship between disease activity (ASDAS) and time in 51 patients (**A**: ASDAS-CRP, **B**: ASDAS-ESR).
The percentage of inactive (ASDAS<1.3) and moderate (1.3≤ASDAS<2.1) disease activity increased as treatment was continued, while the percentage of high (2.1≤ASDAS≤3.5) and very high (ASDAS>3.5) disease activity decreased dramatically from baseline to week 16.

Table III. Baseline biomarkers of patients and healthy controls.

Biomarkers (pg/ml)	Patients (n=20)	Controls (n=20)	*p*-value*
BAFF	2606.31 (2103.95-3647.24)	2195.01 (1807.07-2653.74)	0.028
Chitinase-3-like 1	445.16 (368.58-664.77)	319.46 (247.63-319.46)	0.006
IFN-γ	4.56 (3.87-7.06)	3.86 (3.16-4.56)	0.006
IL-20	4.46 (3.26-6.23)	2.94 (1.90-4.24)	0.018
IL-35	23.06 (14.63-33.21)	12.57 (5.81-19.17)	0.002
MMP-1	1477.70 (968.67-1678.11)	616.24 (392.38-913.58)	0.001
MMP-3	5762.28 (3832.18-7448.39)	3264.12 (2254.99-4639.22)	0.003
Osteocalcin	552.04 (362.78-657.55)	347.57 (266.87-488.34)	0.033
Osteopotinin	9570.26 (6958.64-11043.17)	5181.34 (3743.44-7061.28)	0.001
Pentraxin-3	208.21 (160.70-337.59)	89.70 (68.76-153.29)	0.000
TSLP	20.10 (17.42-28.57)	13.15 (10.63-14.81)	0.000

*All *p*-values are significant.

Table III shows 11 of the 37 cytokines were significantly higher in patients than those in the controls. Positive linear correlations between the biomarkers showed a consistency of the inflammatory cytokines. At baseline, the inflam matory biomarkers had positive correlations with clinical parameters, such as ASDAS, BASDAI, CRP and ESR.

First stage outcomes

After the first stage of 3 courses of ^{99}Tc-MDP treatment, clinical parameters had extraordinary changes (Ta-

Table IV. Comparison of clinical parameters between baseline and week 16.

Assessment	Baseline (n=51)	Week 16 (n=51)	*p*-value
ASDAS-CRP	3.66 ± 0.83	2.68 ± 0.92	<0.001¥
ASDAS-ESR	3.22 ± 0.79	2.33 ± 0.91	<0.001¥
BASDAI	4.53 ± 1.92	3.06 ± 2.11	<0.001¥
BASFI	3.10 ± 2.16	2.45 ± 1.88	<0.001¥
PGA	6.66 ± 2.37	4.11 ± 2.41	<0.001¥
spine pain at night	5.36 ± 2.66	3.00 ± 2.18	<0.001¥
spine pain overall	5.59 ± 2.76	3.38 ± 2.26	<0.001¥
BASMI	5.98 ± 2.23	5.18 ± 2.30	<0.001¥
CRP (mg/L)	26.62 ± 24.60	18.78 ± 19.51	<0.01¥
ESR (mm/H)	28.92 ± 25.13	21.51 ± 18.96	<0.00¥
Morning stiffness (min)	36.84 ± 39.55	19.73 ± 26.24	<0.00¥
Peripheral joints pain	2.57 ± 2.70	2.44 ± 2.44	>0.05

¥ Numbers are significant.
ASDAS: Ankylosing Spondylitis Disease Activity Score; BASDAI: Bath Ankylosing Spondylitis Disease Activity Index; BASFI: Bath Ankylosing Spondylitis Disease Functional Index; PGA: visual analogue scale of patient global assessment of disease activity; BASMI: Bath Ankylosing Spondylitis Metrology Index; CRP: C-reaction protein; ESR: erythrocyte sedimentation rate.

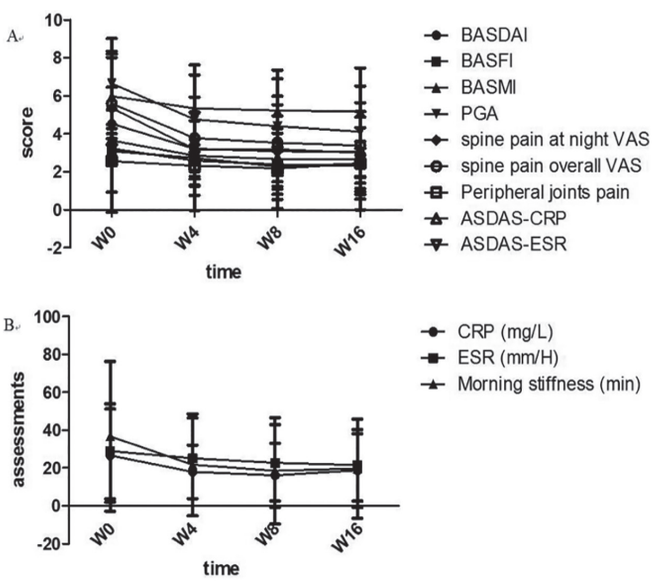

Fig. 3. Longitudinal changes of clinical parameters in 51 patients. Each of the parameters decreased gradually from baseline to week 16.

Table V. Comparison of clinical parameters between baseline and week 30.

Assessment	Baseline (n=27)	Week 30 (n=27)	*p*-value§
ASDAS-CRP	3.76 ± 0.88	2.37 ± 0.96	<0.001
ASDAS-ESR	3.34 ± 0.90	2.04 ± 0.88	<0.001
BASDAI	4.79 ± 2.14	2.25 ± 1.90	<0.001
BASFI	2.93 ± 2.34	1.90 ± 1.62	<0.01
PGA	6.69 ± 2.24	3.00 ± 2.20	<0.001
Spine pain at night	4.73 ± 2.81	1.91 ± 1.78	<0.001
Spine pain overall	5.41 ± 2.83	2.31 ± 1.99	<0.001
BASMI	5.93 ± 2.32	4.41 ± 2.52	<0.001
CRP (mg/L)	28.10 ± 27.27	14.77 ± 11.83	<0.01
ESR (mm/H)	32.33 ± 31.74	19.74 ± 16.58	<0.01
Morning stiffness (min)	37.00 ± 37.45	16.74 ± 24.63	<0.001
Peripheral joints pain	2.86 ± 3.38	1.41 ± 1.83	<0.05

§ All *p*-values are significant.
ASDAS: Ankylosing Spondylitis Disease Activity Score; BASDAI: Bath Ankylosing Spondylitis Disease Activity Index; BASFI: Bath Ankylosing Spondylitis Disease Functional Index; PGA: visual analogue scale of patient global assessment of disease activity; BASMI: Bath Ankylosing Spondylitis Metrology Index; CRP: C-reaction protein; ESR: erythrocyte sedimentation rate.

ble IV). For ASAS improvement, 32 (62.75%) patients got ASAS20 improvement, and 16 of them reached ASAS40; 13 and 25 patients had ASAS partial remission and ASAS5/6 improvement respectively; while 13 (25.49%) patients got no improvement. For ASDAS, ASDAS-CRP was significantly decreased at Week 16, 24 (47.06%) patients reached clinically important improvement (ΔASDAS-CRP≥1.1), and 8 patients got major improvement (ΔASDAS-CRP≥2.0). ASDAS-ESR had the same results. Figure 2 showed the distribution of disease activity changed in the first stage as the patients received treatments.

The clinical parameters (except for peripheral joints pain) improved significantly (Table IV). Figure 3 showed the parameters decreased gradually from baseline to week 16.

After week 16 visit, 32 out of 51 patients entered the second stage according to ASAS20 improvement. However, 5 (15%) patients were excluded before week 30 because of complications (n=1), economic pressure (n=2) and non-compliance (n=2). The characteristics of patients excluded did not differ from that of patients included at week 30. At last, 27 patients, with a mean age of 37.04±9.41 years, completed the second stage and reached the end point of the follow-up period.

Second stage outcomes

At week 30, the end point of the investigation, 27 patients all achieved ASAS20 improvement, and 13 (48.15%) patients reached ASAS40, 10 and 21 patients had ASAS partial remission and ASAS 5/6 improvement, respectively. For ASDAS, ASDAS-CRP was significantly reduced at Week 30 (Table V), 18 (66.67%) patients reached clinically important improvement, and 8 patients achieved major improvement. ASDAS-ESR also had the same results. Figure 4 showed the distribution of disease activity changed as the patients received treatments. Generally, the percentages of improvements of ASAS and ASDAS at week 30 were greater than those at week 16.

All clinical parameters significantly improved at week 30 (Table V). The

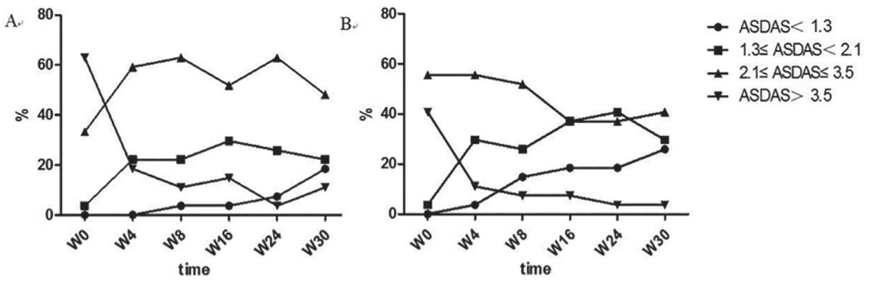

Fig. 4. Longitudinal relationship between disease activity (ASDAS) and time in 27 patients (**A**: ASDAS-CRP, **B**: ASDAS-ESR).
The percentage of inactive (ASDAS<1.3) and moderate (1.3≤ASDAS<2.1) disease activity increased as treatment was continued, while the percentage of high (2.1≤ASDAS≤3.5) and very high (ASDAS>3.5) disease activity decreased dramatically from baseline to week 30.

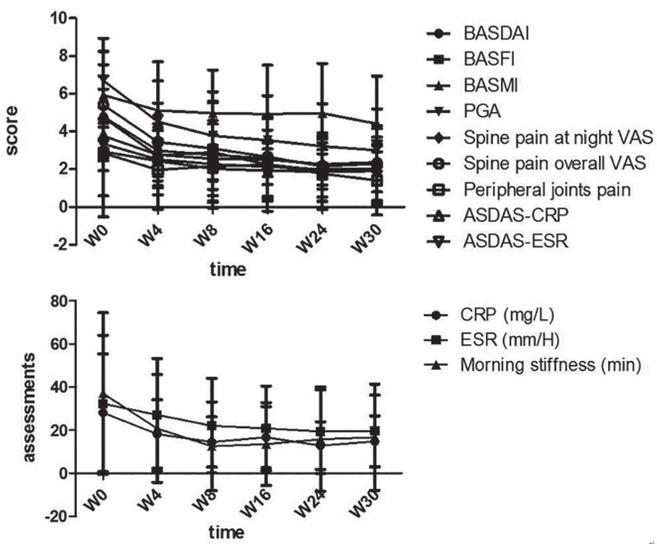

Fig. 5. Longitudinal changes of clinical parameters in 27 patients. Each of the parameters decreased gradually from baseline to the end point.

improvements at week 30 were greater than those at week 16 but the difference was not significant. The improvements of clinical parameters (except for BASMI, morning stiffness time) had significant positive linear correlations with each other, which showed a consistency of the parameters in evaluation. Figure 5 shows how the parameters decreased gradually and significantly from baseline to the end point.

After ^{99}Tc-MDP treatment, 16 biomarkers significantly decreased (Table VI), and the reductions significantly correlated positively with each other. Biomarkers reductions positively correlated with baseline clinical parameters and the improvements of clinical parameters, respectively.

Safety evaluation

Seven (13.73%) adverse events happened in the study, all of them were mild to moderate in intensity. Two of the events were considered possibly related to the treatment (both were liver function damage with hepatitis B virus infected before). No serious adverse event related to the treatment was observed. No patients withdrew from the investigation because of adverse events. No patients had other adverse reactions such as injection reaction or gastrointestinal side-effects. So ^{99}Tc-MDP was considered safe enough for its few side effects and good tolerance in clinical application.

Discussion

This 2-stage (30w-follow-up) clinical study revealed that refractory AS patients were older with long symptom duration and severe structural damage. They also had high disease activity, and their daily function, spine and joint movement were restricted. Statistical analysis showed that older patients with long symptom duration were inclined to have a moderate disease activity, but a limited daily life function.

Refractory AS patients had severe radiological progression, especially in lumbar segment. Statistics revealed that the formation of syndesmophytes tended to occur in older patients with longer symptom duration, rather than in patients with active disease. This means that younger patients with shorter symptom duration usually have severe inflammation but few syndesmophytes. However, the formation of syndesmophyte starts to dominate as the disease progresses by aging and inflammation fades.

After ^{99}Tc-MDP treatment, all of the 27 patients achieved ASAS20 improvement, and two thirds of them reached clinically significant improvement. The percentage of patients with inactive or moderate disease activity increased from baseline to week 16, and increased more at week 30. While the percentage of patients with high or very high disease activity obviously decreased. After 5 courses of treatment, the percentages of patients with ASAS and ASDAS improvement were all increased much more than after 3 courses of treatment. All of the clinical parameters were also significantly improved after treatment, especially at week 30. Accordingly, patients had significant clinical improvement after ^{99}Tc-MDP treatment and the improvement increased as the treatment was continued. The results revealed that if the patients achieved clinical improvement after 3 courses of treatment, it would be recommended for another 2 courses to consolidate the efficacy.

AS patients had high levels of inflammatory biomarkers, which caused inflammation process in AS. After 5 courses treatment, the symptoms and inflammation alleviated as the biomarkers significantly decreased in coincidence. Besides, the reduction of biomarkers was in parallel with the baseline disease activity and the improvement of disease activity, respectively.

The selected 37 inflammatory cytokines had been proved to express elevated levels and contribute to the pathologic process in AS and other inflammatory diseases in various experiments (22-25). According to our investigation,

Table VI. Comparison of biomarkers between baseline and week 30.

Biomarkers (pg/ml)	Baseline (n=20)	Week 30 (n=20)	Reduction	*p*-value[¶]
BAFF	2606.31 (2103.95-3647.24)	2178.83 (1693.71-2687.43)	409.27 (-21.75-830.04)	0.004
sCD30	170.34 (121.51-253.54)	149.22 (103.05-187.42)	35.50 (13.92-84.29)	0.021
sCD163	780.87 (572.53-976.60)	602.21 (510.34-717.66)	119.84 (-11.51-399.63)	0.030
Chitinase-3-like 1	445.16 (368.58-664.77)	329.82 (216.61-447.23)	190.12 (45.28-245.92)	0.001
IFN-γ	4.56 (3.87-7.06)	3.51 (2.43-4.81)	1.24 (-0.69-3.99)	0.020
gp130/sIL-6Rβ	14478.78 (11595.36-16676.33)	11593.27 (9859.12-13597.60)	2499.50 (346.38-4396.71)	0.002
sIL-6Ra	2653.66 (2334.55-3137.92)	2242.28 (1885.93-2520.61)	474.91 (139.99-894.67)	0.003
IL-19	2.43 (1.53-3.45)	0.42 (0.00-2.15)	1.59 (0.11-2.32)	0.003
IL-35	23.06 (14.63-33.21)	15.03 (11.33-19.08)	6.51 (3.16-17.14)	0.002
MMP-3	5762.28 (3832.18-7448.39)	2986.12 (1844.94-4640.34)	2199.62 (339.63-3417.77)	0.002
Osteocalcin	552.04 (362.78-657.55)	378.94 (246.95-604.94)	114.18 (-66.61-265.47)	0.028
Osteopotinin	9570.26 (6958.64-11043.17)	7732.33 (5226.94-9202.35)	2254.01 (-1056.11-3527.58)	0.019
Pentraxin-3	208.21 (160.70-337.59)	147.05 (84.27-196.81)	66.63 (25.40-164.86)	0.002
sTNF-R1	427.43 (297.53-611.52)	303.85 (209.76-494.49)	81.61 (25.37-164.86)	0.001
sTNF-R2	2112.92 (1427.23-3077.21)	1383.19 (878.95-2359.84)	535.92 (28.06-1069.62)	0.004
TSLP	20.10 (17.42-28.57)	16.04 (12.89-18.28)	6.05 (1.88-10.88)	0.002

[¶] All *p*-values are significant.

9 cytokines including BAFF, chitinase-3-like 1, IFN-γ, IL-35, MMP-3, osteocalcin, osteopotinin, pentraxin-3 and TSLP, overexpressed in refractory AS patients, and significantly decreased as symptom alleviated after ^{99}Tc-MDP treatment, which revealed that the biomarkers were relevant to the pathogenesis and clinical aspects of AS. Studies showed that MMP-3 was a biomarker of AS disease activity for it increased in AS and decreased after TNF inhibition, and also correlated with swollen joint count (26, 27). Another study suggested MMP-3 was an independent predictor of radiographic progression in AS (28). Our study also confirmed the proinflammatory effect of MMP-3 in AS. Osteocalcin played an unclear role in AS, for parts of the studies showed there was no difference between AS and controls, but others showed elevated levels in AS patients (26, 29). In our study, osteocalcin was elevated in AS and decreased after ^{99}Tc-MDP treatment, which might suggest osteocalcin had a significant effect in AS pathogenesis. Niederer's study inferred that BAFF was not elevated in AS patients, and a slight increase could be observed following a good response to TNF inhibition (30), which was the opposite of our results, since BAFF increased in AS patients and decreased after ^{99}Tc-MDP treatment. The different results might need further investigation. Feng confirmed that IFN-γ could activate HLA-B27 promoter and induce the unfolded protein response in HLA-B27-expressing cells, then resulted in AS (31). Our study also implied that IFN-γ promoted the AS inflammation process. Li's trial showed serum level of IL-35 was higher in psoriatic arthritis patients than in psoriasis patients and HCs (32), and our study showed it was also higher in AS patients than in controls, which suggested that IL-35 may played an extraordinary function in arthritis disease as an inflammatory cytokine. Levels of pentraxin-3 increased in association with inflammation in patients with AS (33, 34), and our study concluded the same results. Overexpression of sTNF could induce systemic inflammation and bone erosion. It had been clear that sTNF-R1 was a biomarker of inflammation in AS, and decreased after effective treatment (35). However, the effect of sTNF-R2 in AS was not distinguished, because some studies showed sTNF-R2 could enhance AS inflammation but others did not (36, 37). In our study, we confirmed that both sTNF-R1 and sTNF-R2 were not elevated in AS patients, but decreased significantly as symptoms alleviated after ^{99}Tc-MDP treatment, which suggested that the two biomarkers might be pathogenetic factors in AS.

Apart from showing the relevance between biomarkers and the clinical aspects of AS at baseline and after treatment, our investigation also revealed that the reduction of biomarkers was in parallel with the improvement of disease activity. These results are supportive of the hypothesis that the inflammatory cytokines played vital roles in the pathogenesis of AS.

What needs to be specified is that we only evaluated the biomarkers at baseline (week 0) and the end (week 30), for the two points were the important moments of the whole study, and we were able to have obvious feedback after the treatment. Additional detection of the inflammatory cytokines at each visit from week 0 to week 30 is expected, which can provide us with more information about the clinical improvement and the regulation of biomarkers to furthermore show the relevance between the biomarkers and pathogenesis of AS.

Comparison between the efficacy of ^{99}Tc-MDP and other treatments had been taken into consideration. According to ASAS/EULAR recommendations, the therapeutic medication of AS includes NSAIDs, DMARDs and anti-TNF agents (38, 39). NSAIDs are recommended as first-line drug treatment for AS patients with pain and stiffness, and continuous treatment with NSAIDs is preferred for patients with persistently active, symptomatic disease. In DMARDs, only sulfasalazine may be considered in patients with peripheral arthritis. Anti-TNF therapy should be given to patients with persistently high

disease activity. These conventional medications can reduce inflammation, relief discomfort and improve the patients' condition. However, the ASAS/EULAR recommendations have shortcomings. NSAIDs is the first choice to inhibit inflammation in AS treatment, Wanders and Poddubnyy also proved that high NSAID intake over 2 years could retard radiographic spinal progression in AS (40, 41), but the cardiovascular, gastrointestinal and renal risks should be taken into account. Sulfasalazine is a kind of slow medication with uncertain efficacy, and the gastrointestinal side-effects caused by it should be mentioned. TNF inhibition is a revolutionary treatment for most AS patients with outstanding efficacy, but it is relatively expensive for Chinese patients. Osta *et al*. showed that TNF-α had paradoxical effects on bone homeostasis, it can both activate osteoclastogenesis and osteoblastogenesis (42). Meanwhile, many studies revealed that using anti-TNF agents led to resolution of inflammation on magnetic resonance imaging, but radiographic progression was also associated with these drugs (43, 44). So, we can conclude that the limited choices provided by ASAS/EULAR recommendations cannot treat AS patients who were intractable with conventional treatments, and have no effective methods targeting on unbalanced bone turnover either. However, ^{99}Tc-MDP provides us with a new opportunity, especially in refractory AS patients.

^{99}Tc-MDP has low toxicity and a long half-life, it is also high bone-targeted. Most of the previous investigations of ^{99}Tc-MDP are focused on RA with the emphasis on anti-inflammation and bone turnover regulation. Firstly, ^{99}Tc-MDP could decrease the level of IL-1β, TNF-α, PGE_2, IL-1 and IL-6, and then allay inflammation (45-47). Secondly, ^{99}Tc-MDP could reduce the level of RANKL, Dkk-1 and MMP-3, and increase OPG level, to limit bone resorption and promote bone formation (48-51). Hence, besides the effects of inhibiting inflammation, relieving pain and regulating immunity, ^{99}Tc-MDP might also regulate unbalanced bone turnover in RA. In our study, refractory AS patients achieved resolution of inflammation and a decrease of overexpressed inflammatory cytokines after ^{99}Tc-MDP treatment. One limitation of the trial is that we did not explore the biomarkers of bone turnover in AS patients during the treatment. What remains to work out is that, considering ^{99}Tc-MDP can inhibit osteoporosis in RA, it may be able to regulate the pathological bone turnover in AS patients. Therefore, it is worth expanding the application of ^{99}Tc-MDP, and further investigation is expected to uncover the underlying mechanisms and to ensure more improvement in patients.

Finally, ^{99}Tc-MDP was safe in clinical application with only 13% adverse events.

In conclusion, it was the first clinical trial that concentrated on evaluating the efficacy and revealing inflammatory mechanisms of ^{99}Tc-MDP in refractory AS patients. This study showed that refractory AS patients are older with long disease duration and severe structural damage, they also had high levels of disease activity and inflammatory biomarkers. Patients achieved significant clinical symptom and inflammation improvement as the biomarkers significantly decreased after ^{99}Tc-MDP treatment and the improvement increased as the treatment continued. Therefore, consolidated treatment of ^{99}Tc-MDP proved to be safe, effective and is recommended in clinical application.

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