Cervus and cucumis peptides combined umbilical cord mesenchymal stem cells therapy for rheumatoid arthritis

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Abstract

Cervus and cucumis peptides (Lugua polypeptides, LG) are traditional Chinese medicine, which are active components of polypeptide extracted from Sika deer bone and melon seed, and they contain bone induced polypeptide biological factors. Umbilical cord mesenchymal stem cell (UC-MSC) have tissue repair multiple effects, anti-inflammatory, and immune regulation function, which become a very promising start in rheumatoid arthritis (RA) treatment. Hence, LG combined UC-MSC can significantly enhance the UC-MSC treatment of rheumatoid arthritis (RA).

To explore the clinical curative effect and therapeutic mechanism of LG combined UC-MSC for treating RA.

119 patients were divided into control and treatment groups, and both groups were treated with methotrexate tablets, lefunomide, and UC-MSC. But, LG were added to the treatment group. In vitro, the effects of LG on UC-MSC cell secretion of anti-inflammatory factors were also performed.

The Health Assessment Questionnaire; the 28 joint disease activity score; C reactive protein; the erythrocyte sedimentation rate; rheumatoid factor; and anti-cyclic citrullinated peptide antibody were significantly reduced in treatment group 1 year after treatment ($P < .05$). In vitro, compared with the control group, the number of hepatocyte growth factor (HGF), the secretion of prostaglandin E2 (PGE2) and tumor necrosis factor-inducible gene 6 protein (TSG6) increased significantly ($P < .05$).

LG combined UC-MSCs can significantly improve the curative effect of RA patients, while LG may reduce inflammatory cytokines, regulate immunity, improve microcirculation, and are conducive to UC-MSCs migration and the repair of damaged tissue.

Abbreviations: Anti-CCP = anti-cyclic citrullinated peptide antibody, CRP = C reactive protein, DAS 28 = the 28 joint disease activity, ESR = the erythrocyte sedimentation rate, HAQ = the Health Assessment Questionnaire, LG = cervus and cucumis peptides, RA = rheumatoid arthritis, RF = rheumatoid factor, UC-MSC = umbilical cord mesenchymal stem cell, WBC = white blood cells.

Keywords: anti-inflammatory cytokines, deer and melon Polypeptides, mesenchymal stem cells, repair damage tissue, rheumatoid arthritis

1. Introduction

rheumatoid arthritis (RA), an autoimmune disease, is characterized by inflammatory synovitis, which can cause complete joint damage, as well as extra-articular and generalized body symptoms. Without timely diagnosis and treatment, these symptoms may eventually lead to disability and premature death.[1,2] So, exploring new and effective treatments for RA is pressing issues for medical personnel in the rheumatic field. Although many studies[3-5] have showed that RA is closely related to the expression of inflammation gene, the pathogenesis
of RA is still unclear.\[6\] In the past 2 decades, various new biological agents and the optimization of treatment principles and strategies, were executed in the treatment of RA, and the prognosis of patients were significantly improved.\[17\] However, due to the side effects of drugs and other reasons, various treatments have their limitations, and for some patients, the curative effect are not satisfactory. Therefore, it is urgent to explore new therapeutic methods through their mechanism of action.\[18\]

Umbilical cord mesenchymal stem cells (UC-MSCs) have tissue repair, multiple effects, anti-inflammatory and immune regulation function, which become a promising therapy method in RA therapeutic areas.\[9\] Many studies have shown that UC-MSCs therapy for RA can safely, effectively and persistently alleviate the RA symptoms.\[10\] Our preliminary study\[11\] also reported that UC-MSCs had very good safety and effectiveness in the treatment of RA, and the patient’s tolerance was good.\[12\] In addition, cervus and cucumis peptides (LG) is a compound preparation,\[13\] which is the active component of polypeptides extracted from the bones of the sika deer and the seeds of melon. It contains bone-induced polypeptide biological factors, such as transforming growth factor-beta (TGF-beta),\[14\] which can promote the synthesis of type I collagen and osteoporosis.\[14\] They inhibit the inflammatory response, reduce the content of tumor necrosis factor-alpha (TNF-alpha) in serum,\[15\] and assist macrophage-infiltration.\[16\] According to the pathogenesis of RA in traditional Chinese and western medicine, UC-MSCs are pre-stimulated by the polypeptide of LG to promote the superposition of kidney-nourishing, bone strengthening, blood-activating, and collateral-opening, and also reduce the release of pro-inflammatory factors. While stabilizing the internal environment, the biological characteristics of UC-MSCs migration, homing, regeneration, repair, and immune regulation can be enhanced, which also conform to the principle of specimen co-treatment in traditional Chinese medicine.

Base on the above mechanism, we hypothesized that LG early intervention in UC-MSCs can significantly increase the effective treatment of RA. However, the specific mechanism was not clear. In this study, we observed the clinical effect of LG combined with UC-MSCs in the treatment of RA patients and studied the potential mechanism of LG to enhance the effectiveness of UC-MSCs therapy for RA, In addition, through a vitro-cell model, we focused the synergistic effect of LG on the secretion of anti-inflammatory factors to UC-MSCs.

2. Materials and methods

2.1. Patients selection

All 119 RA patients were selected from January 2011 to December 2018, and the inclusion criteria referred to the American College of Rheumatology (ACR) standard.\[17\] Patients were randomly divided into LG + UC-MSCs group (n = 59) and UC-MSCs group (n = 60). There were 6 males and 53 females in the treatment group with an average age of 43 years, and the average time of illness course was 10 years. There were 7 males and 53 females in the control group, with an average age of 44 years and the average time of illness course was 10 years (Table 1). The exclusion criteria were following: pregnant or lactating women; complicated with serious primary diseases of cardiovascular, liver, kidney and blood system; mentally ill; patients with skin diseases or skin lesions. And this study was registered in ClinicalTrials.gov (NCT01547091).

2.2. Treatment protocol

For LG + UC-MSCs group, all patients were firstly administered intravenous drip with 100 mL 0.9% sodium chloride mixed with 24 mg LG injection (Harbin Yuheng pharmaceutical LTD, China); 1 time/d, totally 7 days and oral 5 mg methotrexate, 2 times/wk, oral 10 mg leflunomide, 1 day, then administered intravenous drip with 40 mL UC-MSC cells (4 × 10^7/40mL) (Shenzhen Bio LTD, China.). Patients in the UC-MSCs group were administrated oral 5 mg methotrexate, twice a week, oral 10 mg leflunomide, once a day, and 40 mL UC-MSC cells (4 × 10^7/40mL).

2.3. Clinical effect evaluation

Based on our previous study,\[18\] the 28 joint disease activity score (DAS 28), the health assessment questionnaire (HAQ) score and RA clinical remission standard (ACR standard) were recorded 1 year before and after treatment.\[19\] The erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), rheumatoid factor (RF), and anti-cyclic citrullinated peptide antibody (Anti-CCP) were detected pretreatment and 1 year after treatment. The routine hematuria and liver and kidney functions were detected pretreatment and 1 year after treatment to evaluate adverse reactions and safety during treatment.

2.4. In vitro experiment

Experimental materials: LG injection (Harbin Yusheng pharmaceutical LTD), Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) and fetal calf serum (FBS) (Invitrogen, USA). Automatic enzyme marker (Thermo Fisher, USA). Hepatocyte growth factor (HGF), prostaglandin E2 (PGE2) and tumor necrosis factor-inducible gene 6 protein (TSG6) enzyme-linked immunosorbent assay (ELISA) Kit (Nanjing Jincheng Biological LTD, China). The UC-MSC cells (Tianjin Heze Bio LTD, China.) were cultured in DMEM/F12 medium containing 10 ng/mL Fibroblast growth factor-basic, (bFGF) (Gibco, USA), 10% FBS and 1% penicillin/streptomycin at 37.0°C, 5% CO2 and 95% humidity.

Experimental method: When the UC-MSC cells were growing in good condition, then they were inoculated in 4 wells in 6-well
plates overnight. All medium were discarded and then we added medium containing 0ug/mL, 3ug/mL, 6ug/mL and 9ug/mL LG injection, respectively, for co-culturing 48 hours. The supernatant were collected and centrifuged at 3000 rpm for 20 minutes, then stored for ELISA detection. Blank well, standard well and sample well were set for testing. The blank holes were used to adjust to zero, and the absorbance value of each well was measured at the wavelength of 450 nm.

2.5. Statistical analysis
SPSS statistics 21.0 (IBM) software was used for data statistical processing. The measurement data were presented as mean ± standard deviation (x ± s), and t-test was used for analyzing data, and P < .05 was considered statistically significant. The statistical analysis and figure plots were performed using GraphPad software (Prism 8 version, San Diego, CA).

3. Results
3.1. Curative effect evaluation
The comparison of DAS28 scores and HAQ scores of the 2 groups before and after treatment were shown in Table 2. The DAS28 scores and HAQ scores of 2 groups after treatment were both decreased significantly than those of pretreatment. What's more, the other immunology factors such as CRP, ESR, RF, and Anti-CCP had no significant difference in the UC-MSCs group, but they decreased significantly in the LG+UC-MSCs group.

3.2. Curative safety evaluation and adverse reactions
All the 59 patients in the LG+UC-MSCs group conformed the ACR20 index 3 months after treatment, and of which 14 cases conformed the ACR50 index. In the UC-MSCs group, 30 cases conformed the ACR20 index, of which seven cases conformed the ACR50 index. In the LG+UC-MSCs group after the infusion of LG combined UC- MSC, only 2 patients had gastrointestinal symptoms of different degrees. Six cases had mild leukocytopenia (white blood cells [WBC] < 3.5 × 10⁸). Among the 60 patients in the UC-MSCs group, 16 patients had adverse reactions, among which six had gastrointestinal symptoms of different degrees, 1 had moderate leukocytopenia (WBC < 2.0 × 10⁸), six had mild leukocytopenia (WBC < 3.5 × 10⁸), and 3 had mild transaminase elevation.

Routine laboratory indicators had no significant change during the pretreatment and 1-year posttreatment between UC-MSCs and LG+UC-MSCs group (P > .05). Routine hematuria and liver and kidney function tests were all normal 1 year after treatment (Table 3).

3.3. LG promoted the secretion of anti-inflammatory factors of UC-MSC
In the ELISA test, the LG treated UC-MSC cells group significantly increased the secretion of HGF, PGE2, and TSG6 more than the UC-MSC cells group. Fig. 1 showed that LG could promote the secretion of anti-inflammatory factors of UC-MSC in a concentration-dependent manner (P < .01).

### Table 2
Comparison of clinical indicators change between the 2 groups before and after treatment.

|                | UC-MSCs Pretreatment | Posttreatment | P value | LG + UC-MSCs Pretreatment | Posttreatment | P value |
|----------------|-----------------------|---------------|---------|---------------------------|---------------|---------|
| DAS28          | 5.82 ± 1.4            | 3.96 ± 1.09   | .011    | 6.05 ± 1.7               | 3.55 ± 1.40   | .023    |
| HAQ            | 2.51 ± 2.07           | 3.45 ± 1.21   | .024    | 1.99 ± 1.17               | 1.11 ± 1.29   | .019    |
| CRP (µL/L)     | 109.44 ± 37.09        | 27.13 ± 23.02 | .687    | 33.16 ± 25.37             | 18.67 ± 19.01 | .032    |
| ESR (mm/h)     | 65.07 ± 32.67         | 45.97 ± 22.91 | .071    | 52.22 ± 45.12             | 21.89 ± 14.53 | .005    |
| RF (µL/mL)     | 251.04 ± 65.98        | 198.08 ± 95.50| .009    | 323.01 ± 95.42            | 239.07 ± 85.58| .018    |
| AntiCCP (µL/mL)| 251.04 ± 89.98        | 204.82 ± 84.05| .159    | 356.03 ± 112.03           | 280.22 ± 110.01| .044    |

P value represents that the compare between pretreatment and posttreatment.

### Table 3
Routine laboratory indicators change between the 2 groups before and after treatment.

|                | LG + UC-MSCs Pretreatment | Posttreatment | P value | UC-MSCs Pretreatment | Posttreatment | P value |
|----------------|---------------------------|---------------|---------|-----------------------|---------------|---------|
| TP (g/L)       | 63.77 ± 4.05             | 62.99 ± 5.25  | .008    | 60.22 ± 4.76          | 62.01 ± 4.72  | .089    |
| Albumin (g/L)  | 108.58 ± 12.52           | 110.50 ± 11.89| .122    | 125.01 ± 12.76        | 117.50 ± 10.54| .411    |
| Platelet (10⁸/µL)| 182.36 ± 70.46          | 182.20 ± 65.02| .368    | 190.02 ± 56.55        | 190.20 ± 55.76| .432    |
| WBC (10⁹/µL)   | 6.31 ± 2.07              | 6.02 ± 2.55   | .087    | 7.01 ± 3.02           | 6.95 ± 2.46   | .285    |
| RBC (10¹³/µL)  | 3.72 ± 0.44              | 3.83 ± 0.59   | .232    | 4.07 ± 0.88           | 4.16 ± 0.74   | .337    |
| BUN (mmol/L)   | 4.80 ± 1.47              | 4.39 ± 1.55   | .467    | 4.20 ± 1.66           | 4.89 ± 2.04   | .535    |
| Triglyceride (umol/L) | 42.31 ± 11.26         | 43.52 ± 11.79| .593    | 43.11 ± 12.08         | 43.39 ± 10.89| .116    |
| ALT (IU/L)     | 20.42 ± 5.40             | 19.70 ± 10.36| .292    | 21.07 ± 5.77          | 19.88 ± 11.05| .557    |
| AST (IU/L)     | 29.42 ± 5.44             | 29.79 ± 12.26| .535    | 30.28 ± 4.97          | 32.06 ± 11.75| .820    |
| Cholesterol (umol/L)| 43.11 ± 11.36        | 43.12 ± 10.55| .191    | 40.55 ± 10.89         | 42.11 ± 9.57  | .223    |

The data are shown as x ± s. P value represents that the compare between pretreatment and posttreatment.

ALT = alanine aminotransferase, AST = aspartate aminotransferase, BUN = blood urea nitrogen, RBC = red blood cells, TP = total protein, P value represents that the compare between pretreatment and posttreatment, WBC = white blood cells.
Mesenchymal stem cells (MSCs) have biological characteristics of regenerating and repairing parenchymal tissues and organs and immune regulation. Although the clinical treatment of RA has made breakthroughs, there are still some problems to be solved. One is that RA joint injuries still occur, which is not only associated with inflammation, and that anti-inflammatory therapy alone may not be enough to prevent RA progression. MSCs has shown exciting therapeutic prospects, especially for RA patients with poor response to current treatment.[21] MSC plays a therapeutic role in RA mainly incell differentiation and immune regulation. In the former, MSCs can be differentiated into damaged cells (such as osteocytes and chondrocytes) and thus they play a direct role in repair. Through the latter, MSC can regulate the immune response mediated by T cells, inhibit the production and function of various innate immune cells and play an anti-inflammatory role, regulate immunity and resist tissue injury.[23] Our previous study[18] has shown that UC-MSC therapy for RA can reduce and alleviate the clinical symptoms of RA, and there is no significant change in human blood routine indicators, which showed good safety.

Blood stasis is an important feature of RA understanding in Chinese Traditional medicine (TCM), which leads to the differentiation and maturation of lymphocytes in intestinal related lymphoid tissues and obstructs their homing, and its effect on mucosal immunity is 1 of the pathogenesis of RA.[24] TCM syndrome of kidney deficiency and blood stasis can more accurately and comprehensively reflect the pathological essence of RA.[25] However, since RA is an autoimmune disease caused by multiple factors, it has been recognized by medical rheumatism personnel that the complementary advantages of the combination of traditional Chinese and western medicine together.[24]

Cervus and Cucumis polypeptide (LG) is a new drug of traditional Chinese medicine. Although it is not a therapeutic drug developed for RA, its function mechanism is applied to the pathological RA, so the LG treatment of RA maybe have certain efficacy. The LG are active components extracted from the bones of the sika deer and the seeds of muskmelon.[26] Melon seed extract can improve local capillary permeability, reduce exudation, promote blood circulation, inhibit the release of prostaglandins, and reduce pain. Therefore, LG injection has the effect of tonifying kidney, strengthening bone, removing blood stasis, dispersing the formation, dehumidifying and analgesic.[27] By down-regulating the expression of RA serum inflammatory factors TNF-alpha and IL-23, it can effectively regulate the internal environment of the body and maintain the homeostasis balance of the body.[28] The therapeutic effects of LG on reducing swelling and pain, regulating immunity, and inhibiting bone destruction. A study reported that an adjuvant RA treatment can significantly reduce the number of joint swelling and pain, improve the joint function and state, and increase grip strength, with fewer side effects and good patient compliance. Studies on the modernization of traditional Chinese medicine[29] showed that many traditional Chinese medicines can promote the proliferation and differentiation of MSCs.

We integrated the 2 medicine LG with UC-MSCs on basis of RA pathogenesis in Chinese and western medicine. LG polypeptides stimulated UC-MSCs to reduce the release of a pro-inflammatory factor instability of the internal environment, and increase UC-MSCs immune regulation and regenerative biology, which conformed to the principle of the main pathogenesis of TCM.[23,30] This study showed that the improvement of HAQ, DAS28, ESR, CRP, RF, and Anti-CCP
in the LG+UC-MSC group was significantly better than that in the UC-MSC group at 1 year after treatment (P < 0.05), and all patients conformed the ACR20 standard. This indicated that TCM intervention played an important role in anti-inflammation, regulating immunity and improving microcirculation in the treatment of UC-MSCs, which was beneficial to the migration of UC-MSCs and repair of damaged tissue. The combination of LG intervention UC-MSCs, DMADs and sequential therapy had a synergistic effect on the effectiveness and stability of RA treatment, especially for the remission of clinical symptoms in patients with active RA, and reduced the adverse reactions of DMADs, which was safe and conformed to the treatment principles and strategies of RA.

However, the limits of this study are the following: First, the effect of LG on the immune regulation of UC-MSCs needs to be further verified by the follow-up in vivo studies. Second, the potential mechanism of LG enhances the effectiveness of UC-MSCs in treating RA needs further research. In addition, the underlying molecular mechanism of the LG joint UC-MSCs therapy for RA is needed to clarify.

5. Conclusions

Cervus and Cucumis peptide (LG) combined with UC-MSC can significantly increase the UC-MSC effectiveness for the treatment of RA, which can improve clinical curative effect and reduce side effects. Also, an in-vitro study cell indicated that LG could enhance the immune regulatory function of UC-MSC by promoting the secretion of HGF, PGE2 and TSG6 anti-inflammatory factors in a concentration-dependent manner. Thereby, UC-MSC combined LG synergistic treatment maybe provide novelty, safety and effective method for RA patients.

Author contributions

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