Transplantation of human amnion mesenchymal cells attenuates the disease development in rats with collagen-induced arthritis

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Abstract

Objective

Human amnion mesenchymal cells (hAMCs), isolated from the amniotic membrane of human placenta, are a unique population of mesenchymal stem cells (MSCs). Recent studies indicated that hAMCs had immunosuppressive functions and might be used in treatment of some autoimmune diseases. The aim of this study is to explore the feasibility of using hAMCs for treatment rats with collagen-induced arthritis (CIA), a classic animal model for human rheumatoid arthritis.

Methods

SD rats were immunised with type II collagen and Freund's incomplete adjuvant. hAMCs were injected intraperitoneal when arthritis had become established. The arthritis was evaluated macroscopically and microscopically. Serum levels of IFN-γ, TNF-α, SOD, MDA, GSH-Px and T-AOC were detected by commercially assay kits. $CD4^+/CD8^+$ T-cell ratio in peripheral blood was examined by flow cytometry. Proliferation of splenocytes was evaluated using MTT assay.

Results

The results demonstrated that application of hAMCs significantly ameliorated severity of arthritis and decreased the histopathological changes in CIA rats. Consistently, production of proinflammatory cytokines such as IFN-γ and TNF-α was dramatically inhibited. Moreover, hAMCs exerted anti-oxidative capacity by significantly raising the levels of SOD, GSH-Px, T-AOC and lowering the level of MDA. In addition, hAMCs also remarkably restored $CD4^+/CD8^+$ T-cell ratio and induced hyporesponsiveness of T lymphocytes by inhibiting their active proliferation. Finally, hAMCs had no obvious side effect on CIA rats.

Conclusion

In conclusion, our results indicated that hAMCs could attenuate the disease development in rats with CIA, which might be a promising cell source for therapy of rheumatoid arthritis.

Key words

human amnion mesenchymal cells, rheumatoid arthritis, immunosuppression

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Introduction

Rheumatoid arthritis (RA) is a chronic, systemic, immune-mediated inflammatory disease, characterised by chronic inflammation and synovial hyperplasia leading to cartilage and bone destruction (1). Apart from many key free radicals and inflammatory cytokines, some immune cells such as activated T cell and macrophages have been proved to participate in the pathogenesis of RA (2-4). Until now, although some new biological agents such as TNF-α inhibitors and IL-1 antagonists emerged, there are still no cures for RA. Novel approaches for treatment of RA are still urgently needed.

Mesenchymal stem cells (MSCs) are multipotent progenitor cells that can be isolated from many different tissues, including bone marrow, adipose tissue, and umbilical cord etc (5). Besides their differentiation potential, MSCs have also been shown to exert immunosuppressive effects. Therefore, some MSCs have been used in the treatment of graft-versus-host disease and autoimmune diseases such as insulin-dependent diabetes mellitus and RA (6-8). At present, the most common source of MSCs is bone marrow. However, there are some limitations of using bone marrow-derived MSCs in clinic, such as the difficulty of obtaining sufficient cell numbers and the decline of differentiating potential with age (9).

Human amnion mesenchymal cells (hAMCs) are isolated from the amniotic membrane of human placenta. Because they are easily obtained and relatively exempt from ethical problem, hAMCs therefore become a new ideal MSCs resource for clinical application (10). Recent studies have indicated that hAMCs also had immunosuppressive functions, such as blocking maturation of monocytes into dendritic cells (DCs), inhibiting macrophage migration and natural killer (NK) cell-mediated lytic activity (11, 12). These results indicate that hAMCs may have potential clinical use in treatment of some autoimmune diseases. Until now, application of hAMCs in the treatment of multiple sclerosis, spinal cord injury and liver fibrosis have been successfully done with significantly immunosuppressive effects (13-15). However, utilisation of hAMCs for the treatment of experimental arthritis has still not been explored. Collagen induced arthritis (CIA) in rats is a classical animal model for the investigation of RA in humans, and some studies have successfully used this animal model to evaluate efficacy of drugs (16, 17). Therefore, in the present study, we investigate the effect of hAMCs in the treatment of rats with CIA.

Materials and methods

Isolation of hAMCs

Placentas were obtained at elective cesarean section with informed consent. hAMCs were isolated from abandoned human placentas according to our previously described with some modifications (10). In brief, amnion layer was mechanically peeled off from chorion layer and washed several times with Hanks' balanced salt solution (HBSS) without calcium and magnesium to remove blood as completely as possible. Then, the amnion was digested with 0.25% trypsin (Gibco BRL, Gaithersburg, MD, USA) at 37°C for 30 min. Further, the amnion was cut into pieces of 1 cm^2 and digested with 0.1% collagenase V (Sigma-Aldrich, St. Louis, MO, USA; dissolved in DMEM/F12) at 37°C for additional 30 min followed with centrifugation (1500 rpm, 10 min) and thorough washing with phosphate buffer saline (PBS). Finally, separated hAMCs were cultured in DMEM/F12 supplemented with 10% fetal bovine serum (Gibco BRL, Gaithersburg, MD, USA). hAMCs of no more than 3 passages were used for experiments. In addition, all the correlated ethical issues concerning this study were approved by corresponding institutional review board (IRB number: CJFH001035).

Rats

Male Sprague-Dawley (SD) rats, with a weight of 180±10g, were purchased from the Institute of Experimental Animals in Chinese Academy of Medical Science (The Rodent License No. SYXK 11-00-0039). The rats were housed under standard laboratory conditions, and food and tap water were provided *ad libitum*. All procedures

using rats were approved by the Ethical Animal Care and Use Committee, China Academy of Chinese Medical Sciences.

Induction and assessment of CIA

The induction and assessment of CIA were performed as previously described (18, 19). Briefly, male SD rats were intradermally injected at the base of tail with 100μg bovine type II collagen (Sigma-Aldrich, St. Louis, MO, USA) in 0.05M acetic acid emulsified with equal incomplete Freund's adjuvant. Seven days after the primary immunisation, the rats were boosted with the same preparation in the same way. Starting from 3 days after the boost immunisation, the degree of arthritis was examined every 2 days. The severity of arthritis was expressed as mean arthritic index on a 0-4 scale according to the following criteria: 0 = no oedema or swelling, 1 = swelling and erythema of the digit, 2 = slight oedema and erythema limited to the foot and /or ankle, 3 = slight oedema and erythema from the ankle to the tarsal bone, 4 = severe oedema and erythema from the ankle to the entire leg. Each posterior limb was graded and thus the maximum possible score was 8 for each animal. A rat with a score of 1 or more than 1 was regarded as arthritic.

Administration of hAMCs

The experimental groups were as follows: (1) normal control (n=6); (2) CIA model control (n=6); (3) CIA rats with hAMCs treatment (n=6). Rats in hAMCs group were intraperitoneal injected with 500μl of cell suspension containing 5×10^6 hAMCs 15 days after the primary immunisation when arthritis had become established (arthritis score ≥1). As a control, rats in normal group and model group were treated with the same volume of saline, injected intraperitoneally. Animals were sacrificed at 21 days after treatment; spleen, liver, kidney, limbs, peripheral blood mononuclear cells (PBMCs) and serum were separated for further studies.

Cytokine assay

Concentrations of IFN-γ and TNF-α in serum were tested using rat IFN-γ and TNF-α ELISA kit (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions.

Detection of SOD, MDA, GSH-Px levels and T-AOC in serum

Levels of super oxide dismutase (SOD), malondialdehyde (MDA), glutathione peroxidase (GSH-Px) and total antioxidant capacity (T-AOC) in serum were determined using commercially available assay kits (Nanjing Jiancheng Bioengineering Institute, China) and were performed according to the manufacturer's recommendations.

Histologic analysis

Formalin-fixed limbs were decalcified and paraffin-embedded using standard histologic techniques. Serial 4μm sections were cut and stained with haematoxylin and eosin to examine morphologic features.

Flow cytometric analysis

PBMCs were isolated by Ficoll (Sigma-Aldrich, St. Louis, MO, USA) and washed twice with PBS. Subsequently, these cells (about 1×10^6) were labelled with FITC-CD3 and PE/CY5-CD4 or FITC-CD3 and PE-CD8 antibodies (Biolegend, San Diego, CA, USA) for 30 min at 4°C. The stained cells were washed and collected using flow cytometry (Becton Dickinson, San Jose, CA, USA) and were analysed using the CellQuest software package (BD Biosciences, San Jose, CA, USA).

Proliferation assay

The spleen was removed and flushed with PBS. Splenocytes were isolated by pressing the spleen between the two frosted slides and passed through 48 μm nylonmesh. Then, the cells were centrifuged once at 260 × g for 5 min and red blood cells were removed by

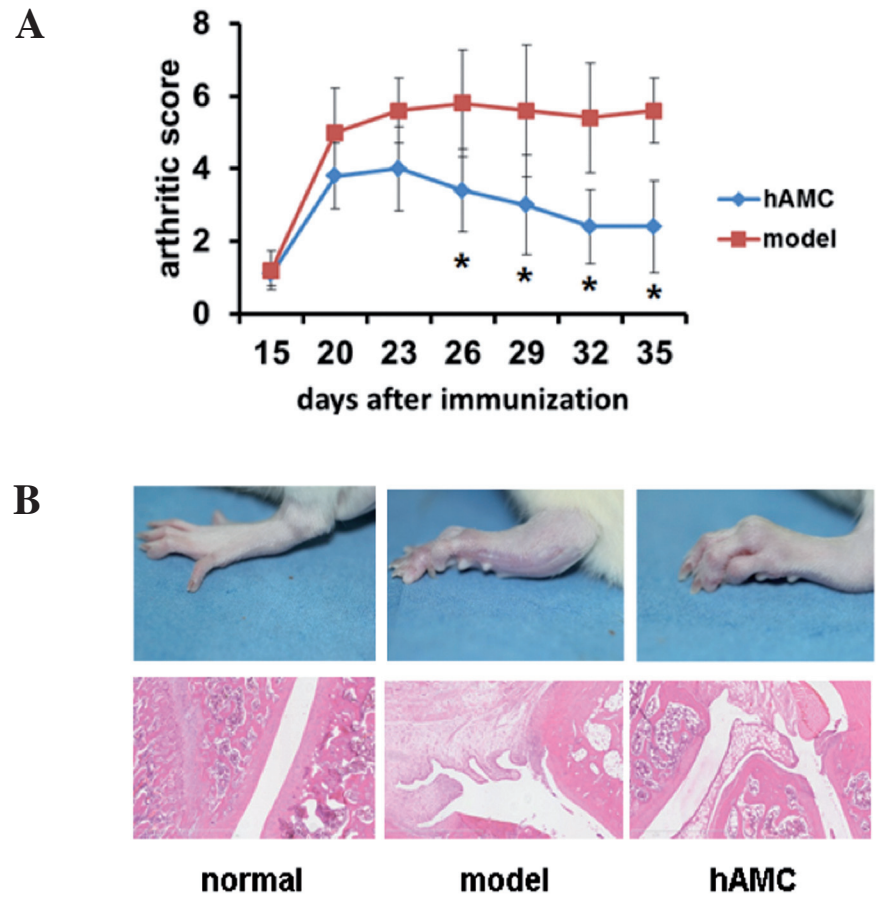


Fig. 1. Effect of hAMCs on arthritic severity of CIA rats.
A: The arthritic scores on different days after immunisation with bovine type II collagen.
B: Morphological and histopathological features of representative ankle joints from different group. H&E stain (×40).

2 ml lysate with 0.84% ammonium chloride. T lymphocytes of splenocytes were isolated from the model control rats and hAMCs-treated CIA rats by negative selection (Miltenyi Biotec, Germany). Cells were plated in a 96-well plate, stimulated with 100μg/ml bovine type II collagen, 10μg/ml ConA, or PBS for 68h. Then, MTT solution (5mg/ml) was added to wells (15μl/well) and incubated at 37°C for 4 additional hours. The reaction was stopped by the addition of 100 μl of lysis buffer (10% SDS) to dissolve the tetrazolium crystals. The plate was examined in a Multiskan Microplate Reader (Thermo Labsystems, Finland).

Statistical analysis

All data were analysed using the SPSS 18.0 statistical package. The data were expressed as mean ± standard deviation (S.D.), and significant differences were assessed using variance test and Student's *t*-test. $p<0.05$ was considered significant.

Results

hAMCs decrease arthritic severity of CIA rats

Animals showed clinical signs of disease starting from nearly 15 days after immunisation, which was consistent with literature reports. Compared with model group, hAMCs treatment could progressively attenuate arthritic severity of CIA rats (Fig. 1A). Further, we verified the therapeutic effects of hAMCs on CIA rats by histological examination at the endpoint of study. As shown in Figure 1B, compared with the normal mice, CIA model mice exhibited severe inflammatory cell infiltration, synovitis, pannus formation, bone and cartilage destruction, whereas these pathological changes had been alleviated in rats treated with hAMCs.

hAMCs inhibit inflammatory cytokine production

To further observe immunosuppressive effect of hAMCs on CIA rats, we detected the serum concentrations of inflammatory cytokines such as TNF-α and IFN-γ, which were regarded as key factors in RA pathological progress. As a result, we found that hAMCs treatment could significantly down-regulate expression of TNF-α and IFN-γ ($p<0.05$) (Fig. 2).

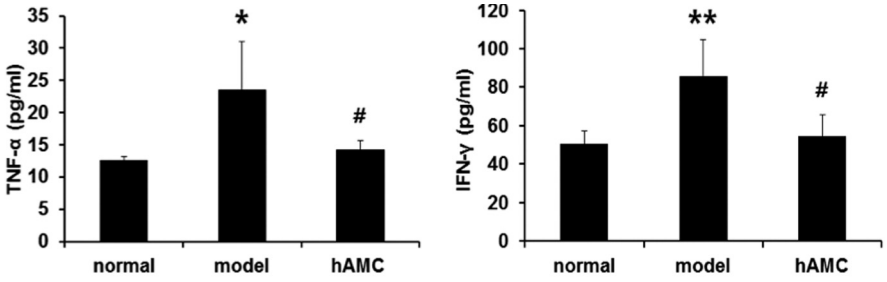


Fig. 2. Effect of hAMCs on TNF-α and IFN-γ production in serum of CIA rats.
The results are shown as the mean±S.D. (n=6). *$p<0.05$, **$p<0.01$, *vs*. normal group; #$p<0.05$, *vs*. model group.

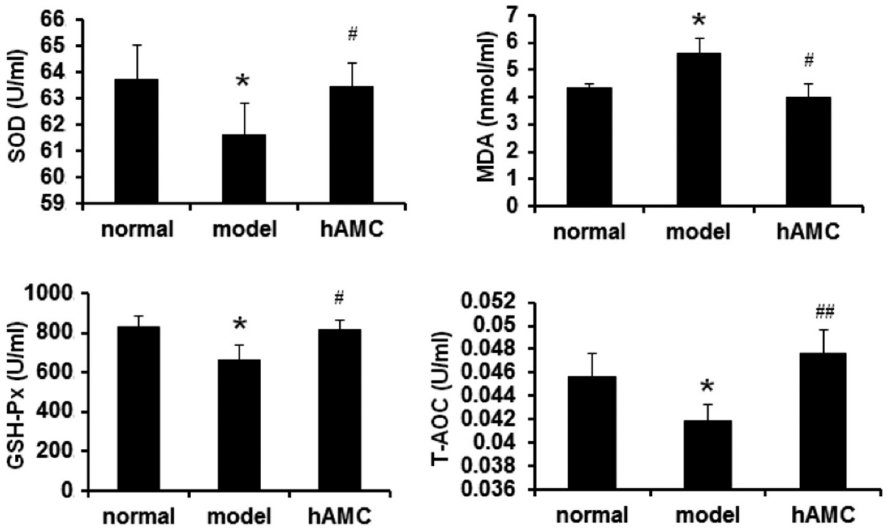
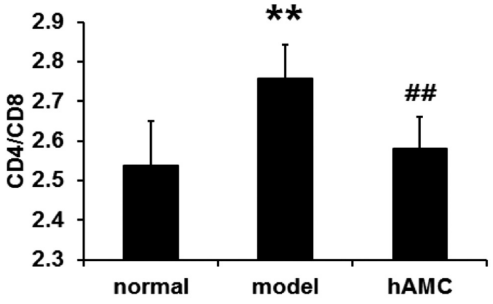


Fig. 3. Effect of hAMCs on SOD, MDA, GSH-Px levels and T-AOC in serum of CIA rats.
The results are shown as the mean±S.D. (n=6). *$p<0.05$, *vs*. normal group; #$p<0.05$, ##$p<0.01$, *vs*. model group.

Fig. 4. Effect of hAMCs on $CD4^+/CD8^+$ T-cell ratio in CIA rats.
The results are shown as the mean ± SD (n=6).
**$p<0.01$, *vs*. normal group; ##$p<0.01$, *vs*. model group.



hAMCs have anti-oxidative capacity in CIA rats

Previous evidence suggests that oxidant stress plays a very important role in the pathogenesis of RA. To investigate whether hAMCs possessed anti-oxidative capacities, we examined the serum levels of SOD, MDA, GSH-Px and T-AOC. As shown in Figure 3, the levels of SOD, GSH-Px and T-AOC were significantly decreased in CIA rats when compared with the normal rats, but the MDA level was greatly increased ($p<0.05$). hAMCs treatment significantly raised the levels of SOD, GSH-Px and T-AOC, at the same time, lowered the level of MDA ($p<0.05$ or $p<0.01$).

hAMCs restore $CD4^+/CD8^+$ T-cell ratio in CIA rats

Studies have proved that $CD4^+/CD8^+$

T-cell ratio was abnormally increased in RA patients. To detect the effect of hAMCs on $CD4^+/CD8^+$ T-cell ratio, we checked the percentage of $CD4^+$ and $CD8^+$ T lymphocytes by flow cytometry. Figure 4 showed that there was higher $CD4^+/CD8^+$ T-cell ratio in the peripheral blood of the CIA rats relative to the normal controls, which was in agreement with other groups' studies. hAMCs treatment could significantly restore the $CD4^+/CD8^+$ T-cell ratio in CIA rats.

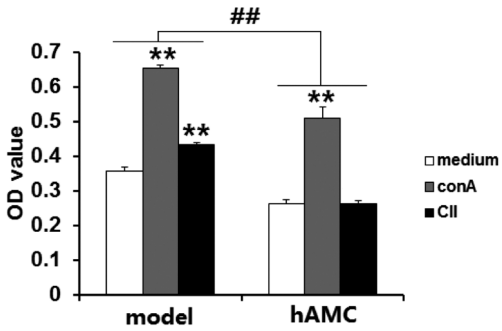
hAMCs induce hyporesponsiveness of T lymphocytes

T lymphocytes from rats that were not treated with hAMCs actively proliferated when stimulated with ConA ($p<0.01$ versus medium) and CII ($p<0.01$ versus medium). T lymphocytes from rats treated with hAMCs also showed basal *in vitro* proliferation, ConA-induced proliferation, and CII-recalled proliferation. However, all of which were significantly reduced compared with proliferation of T lymphocytes from rats that did not receive hAMCs and were cultured under the same conditions ($p<0.01$) (Fig. 5).

hAMCs have no obvious side effect on CIA rats

To investigate whether hAMCs application having side effect on CIA mice, we observed the changes of the body weight, the organ index and the tissue pathology in hAMCs treated rats. We found that compared to the normal animals, CIA rats had lowered body weight. However, this symptom was alleviated in hAMCs treated CIA rats. We then compared the organ index in normal rats, CIA rats and hAMCs treated CIA rats, and found that there were no significantly difference in hepatic index, splenic index and renal index in rats of these three groups (data not shown). Furthermore, histologic assay also show that there were no significantly pathological changes in liver, spleen and kidney tissues in rats of these three groups (Fig. 6).

Fig. 5. Effect of hAMCs on proliferation of T lymphocytes in CIA rats.
C II=type II collagen.
The results are shown as the mean ± SD (n=6).
$^{**}p<0.01$, *vs*. medium;
$^{\#\#}p<0.01$, model group *vs*. hAMCs group.



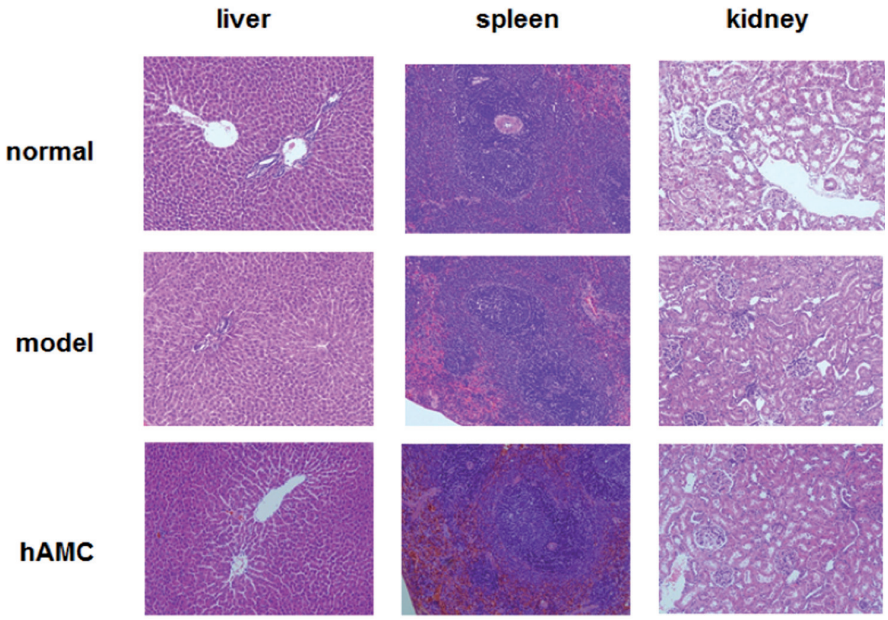


Fig. 6. Histopathological features of liver, spleen and kidney in CIA rats treated with hAMCs.
H&E staining of liver, spleen and kidney tissues at the end of the study (200×).

Discussion

Adult MSCs are non-haematopoietic cells with multilineage potential to differentiate into various tissues of mesodermal origin. Due to this multipotency, they were initially used for tissue engineering and organ transplantation (20). Since immunoregulatory effect of MSCs has recently been discovered, some tries of using MSCs in treating autoimmune diseases have been done and achieved positive results (21). Usually, MSCs are mainly isolated from bone marrow. However, aspirating bone marrow is invasive and sufficient cell numbers are sometimes difficult to obtain for clinic use. Compared with MSCs from bone marrow, hAMCs have several advantages. Firstly, human amniotic membrane is usually regarded as postlabour medical waste and therefore can avoid some ethical problems in clinical application. Secondly, it is easily to obtain large amounts of hAMCs from a single amnion. Thirdly, hAMCs are easily expanded *in vitro* and display stable morphologic characteristics. Finally, using hAMCs in clinic are relatively safe because they do not express telomerase and can avoid risk of tumour formation after cell transplantation (22, 23). Given these characteristics, hAMCs are now considered as an alternative source of stem cells and deserve to be further investigated. Here we showed that administration of hAMCs ameliorated clinical signs and inflammation in CIA rats, at the same time, had no significant side effect, which displayed a therapeutic potential for RA.

Pro-inflammatory cytokines play key roles in the pathophysiology of RA. TNF-α is not only the 'master-regulator' of inflammation in synovium but also contributes significantly to articular destruction (24). Accordingly, it has become a promising target for drug screening (20). Several anti-TNF-α

agents, such as infliximab, etanercept and adalimumab, have been licensed in clinic for treatment of RA. IFN-γ also contributes to inflammation in arthritis. It induces production of cytokines, nitric oxide (NO) and superoxide and expression of MHC class I and class II molecules in macrophages (26, 27). In addition, it also enhances the expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) on endothelial cells, thereby enhancing leukocyte recruitment to the site of inflammation (28). To determine the anti-inflammatory effect of hAMCs on CIA rats, we examined the expression of TNF-α and IFN-γ and found that these two proinflammatory cytokines significantly decreased after hAMCs administration. This result was consistent with our previous *in vitro* experiment in which we found hAMCs could directly lower the production of pro-inflammatory cytokines secreted by monocytes/macrophages (data not shown).

Oxidative damage by oxygen free radicals is an important mechanism in the pathogenesis of RA. In patients with RA, GSH-Px and SOD levels significantly lowered, whereas MDA levels remarkably rose. These results indicated that there was an increase in oxidative stress and a low antioxidant status in RA patients (29). Some researchers even suggested that antioxidant therapy should be co-administrated with conventional drugs to RA patients (30). Recently, several studies reported that MSCs from bone marrow could inhibit oxidative stress (31, 32). In this study, we showed that hAMCs administration significantly decreased MDA level, but increased SOD, GSH-PX and T-AOC levels, which indicated that hAMCs also possessed antioxidant capacity and one mechanism of hAMCs in treating CIA rats might be through anti-oxidation.

A pathogenic hallmark of RA is persistent activation of self-reactive $CD4^+$ T cells (33). In patients with RA, large numbers of $CD4^+$ T cells accumulated in the inflamed synovium (34). At the same time, a high $CD4^+/CD8^+$ T-cell ratio was found in the blood (35). Vaccination against certain TCR Vβ-chains and CTLA4-Ig/LEA29Y treatment showing efficacy in RA further supported the role of T cells in RA pathogenesis (36). Our results demonstrated that hAMCs treatment significantly restored the $CD4^+/CD8^+$ T-cell ratio in CIA rats. Moreover, *in vitro* proliferation rate of T lymphocytes isolated from rats treated with hAMCs was significantly lower than that from model rats. And this phenomenon emerged not only under basal conditions but also when the proliferation was recalled by ConA stimulus or by challenge with the immunising antigen.

In conclusion, our results demonstrated that hAMCs could attenuate the disease development in rats with CIA, which was partly through inhibiting the inflammatory cytokines, decreasing $CD4^+/CD8^+$ T-cell ratio, induced hyporesponsiveness of T lymphocytes and anti-oxidation. Certainly, more studies still need to be done to further explain mechanism of hAMCs and promote their application in clinic.

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