**Methods**

**Animals and Grouping**

In 2019, thirty male Sprague Dawley rats (220±20 g) that were purchased from Experimental and Comparative Medicine Center of Shiraz University of Medical Sciences, Shiraz, Iran were randomly divided into three equal groups of 10 animals. Control group received 0.5 mL/kg of normal saline transrectally after induction of acute colitis. In sulfasalazine group, 500 mg/kg of sulfasalazine dissolved in 0.5 mL normal saline was administered transrectally after inducing acute colitis. In cell treatment group, 2×106 of rat AdSCs of passage 4th in 0.5 mL volume were transplanted transrectally. Acute colitis was induced via enema using a needle inserted up to 8 cm into the colonic tissue and by injection of 2 mL of 3% acetic acid solution, while rats were kept in a vertical position (head-down) to avoid any leakage of acetic acid as described before11. To confirm induction of colitis, rats were checked after 24 hours to suffer from diarrhea or rectal bleeding. Prior to induction of colitis, rats were deprived from food intake for 24 h, but had free access to water.

All treatment interventions were started 24 hours after induction of colitis. During interventions, rats were anaesthetized by intra-peritoneal injection of 3 mg/kg xylazine (2% Rompun, Bayer Co., Germany) and 30 mg/kg ketamine (Imalgène 1000, Merial, Germany). They were kept in separate cages under an ambient temperature of 21±2°C and a 65-70% relative humidity in a good ventilated room. All groups were followed for 21 days. On 7th, 14th and 21st days following treatment measures, the animals were sacrificed, and tissue samples were provided from the distal 10 cm portion of the colon for histological and molecular assessments. The animal studies were undertaken based on NIH Guidelines for the Care and Use of Laboratory Animals. The experimental protocol was approved by the Institutional Animal Care and Use Committee of our University (1398.056). All methods conducted in this study regarding the animals were in accordance with the ethical standards of Institutional Animal Care and Use Committee of Shiraz University of Medical Sciences.

**Isolation of AdSCs**

Adipose tissues around the testes of rats were used for isolation of AdSCs. The isolated tissue was placed on ice under sterile condition and transferred to stem cell laboratory of Stem Cell Technology Research Center of Shiraz University of Medical Sciences, Shiraz, Iran. Briefly, adipose tissue was minced into tiny segments and was treated with 0.2% collagenase type II (Gibco, U.S.A.) for 40 min at 37°C, while shaken. The lysed tissue was centrifuged at 1500 rpm for 5 min. The precipitate was suspended in 2 mL of Dulbecco’s Modified Eagles Medium (DMEM; Gibco, USA) in culture flasks containing 3 mL DMEM supplemented with 10% fetal bovine serum (FBS, Gibco, USA), 1% penicillin and 1% streptomycin (Sigma, USA) and 2 mM L-glutamine (Invitrogen, Netherlands) and were put in an incubator containing 5% CO2 at 37°C and saturated humidity. After 3 days, the media was refreshed to reach 85% confluence. The cells were later sub-cultured until passage 4th using 0.25% trypsin (Gibco, USA), while they were later inactivated by adding equal volume of DMEM.

The isolated AdSCs in passage 4th were later cryopreserved to be used for future cell transplantation purposes using 2×106 viable cells/mL in 50% DMEM media, 40% FBS, and 10% dimethyl sulfoxide (DMSO; MP Bio) in sterile labeled cryovials and kept in a nitrogen tank. To prepare for cell transplantation, they were taken out from nitrogen tank and transferred into a 37°C water bath for thawing. Centrifugation was carried out for 5 min at 1500 rpm and the cell pellet was re-suspended in DMEM and placed in a CO2 incubator at 37°C and saturated humidity until use.

**Characterization of AdSCs by Morphology**

AdSCs were assessed to be morphologically spindle shape.

**Characterization by Osteogenic Induction**

Approximately 5×104 AdSCs were transferred into 6-well plates, while the media was refreshed with osteogenic medium at 90% confluence containing the culture media supplemented with 15% FBS, 100 nM dexamethasone (Sigma-Aldrich, USA), 50 µM ascorbic acid (Merck, Germany), and 10 mM glycerol 3-phosohate (Merck, Germany) for 21 days. The media change was done every 3 days and after 21 days, 10% formalin was added for 20 min to fix the cells. After 3 washes with deionized water, the differentiation was assessed by alizarin red staining (Sigma-Aldrich, USA) bound to calcium mineralized deposits and revealed a red color.

**Characterization by Adipogenic Induction**

Around 5×104 AdSCs were seeded in 6-well plates, while media change happened with adipogenic medium at 90% confluence using culture media supplemented with 15% FBS, 100 nM dexamethasone, 100 µM ascorbic acid, and 200 µM indomethacin (Sigma-Aldrich, USA) for 21 days. After 3 weeks, 10% formalin was added for 20 min to fix the cells and after 3 washes with deionized water, fresh 0.5% Oil Red-O dye (Sigma-Aldrich, USA) dissolved in 2-propanol solution (Merck, Germany) for 2 h was added for staining of differentiated cells. Oil red O staining reveals red color droplets when adipogenic induction is positive.

**Characterization of AdSCs by Flow Cytometry**

CD73 and CD90 (Dako, Denmark) were used to confirm the positive expression of mesenchymal surface markers and CD34 and CD45 (Dako, Denmark) to verify the absence of hematopoietic surface markers.

**Histological Evaluation**

The removed colonic tissue provided from animals was transferred into 10% buffered formaldehyde for 72 h. Then, dehydration was done by cold ethanol, and clearing was undertaken using cold xylene. The tissue samples were later embedded in paraffin at 53°C and a 5-µm thickness tissue section was prepared serially, dried at 37°C for an hour and stained by hematoxylin and eosin (H&E). All slides were visualized under a light microscope and photography was undertaken.

**RNA Extraction of AdSCs**

RNA extraction of AdSCs was done using an RNA extraction kit (Cinna Gen Inc., Tehran, Iran). The RNA was assessed at optical density ratio of A260/A280 and A260/A230 using a Nanodrop spectrophotometer (Nanodrop; Thermo Fisher Scientific,Waltham, USA). cDNA was prepared using 1000 ng total RNA applying the Revert Aid first strand cDNA synthesis kit (Thermo Fisher Scientific, Waltham, USA). Collection of the samples for analyzing the gene expression was based on methods described before12.

**Quantitative Real Time Polymerase Chain Reaction (qPCR)**

The targeted apoptotic genes were Bax and Bcl-2 and B2m was considered as an endogenous control gene. The sequences of these genes were determined by NCBI database and primer sets were designed by primer3 software (Table 1). Real time PCR was carried out using SYBR Green I as reporter dye and Step One Real-Time PCR reactions (Applied Biosystems, Waltham, USA). In each reaction, 200 nM of each primer was added to target the specific sequence. The PCR condition was set at 94°C for 10 min followed by 40 cycles at 94°C for 15 s, at 60°C for 60 s, and melting curve analysis ramping from 65 to 95°C. The amplification signals of different samples were normalized to B2m cycle threshold (Ct), and then the 2-DDCt method was applied to compare mRNA levels of various groups, which represented a fold-change in data analysis12.

**Table 1** The Bax, Bcl-2 and B2m gene sequences designed by primer3 software

|  |  |  |
| --- | --- | --- |
| Gene | Primer sequence | Size (bp) |
| Bax | Forward: 5’-CTGCAGAGGATGATTGCTGA-3’ | 174 |
|  | Reverse: 5’-GATCAGCTCGGGCACTTTAG-3’ |  |
| Bcl2 | Forward: 5’-ATCGCTCTGTGGATGACTGAGTAC-3’ | 134 |
|  | Reverse: 5’-AGAGACAGCCAGGAGAAATCAAAC-3’ |  |
| B2m |  Forward: 5’-**CGTGCTTGCCATTCAGAAA**-3’ |  244 |
|  |  Reverse: 5’**-ATATACATCGGTCTCGGTGG**-3’ |  |

bp: base pair.

**Statistical Analysis**

To compare the groups, one-way analysis of variance (ANOVA), Independent t and Shapiro-Wilks tests and Prism software (GraphPad Software, version 6.0, San Diego, USA) were used. A *P* value < 0.05 was considered statistically significant.