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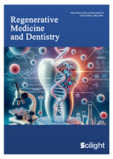

Article

Intravenous Transplantation of Apoptosis Repressor with Caspase Recruitment Domain-Overexpressing Mesenchymal Stem Cells Promotes Bone Formation in Bisphosphonate-Related Osteonecrosis of the Jaw Rats

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Abstract: Bisphosphonate-related osteonecrosis of the jaw (BRONJ) is a serious complication caused by the application of bisphosphonates (BPs) which are widely used in bone metastasis, osteoporosis and other metabolic bone diseases. Since bone marrow-derived mesenchymal stem cells (BMSCs) dysfunction potentially plays a critical role in the development of BRONJ, purposefully improving the function of BMSCs may help reduce the symptoms of BRONJ. Apoptosis repressor with caspase recruitment domain (ARC) can inhibit cell apoptosis and cell death, and was confirmed to possess an obvious reparative function in damaged tissues recently. Therefore, we aimed to investigate whether transplantation of ARC-overexpressing BMSCs had a therapeutic effect on BRONJ and explored possible mechanisms. First, we successfully established the BRONJ rat model and confirmed that BRONJ-derived BMSCs showed decreased proliferation and osteogenic differentiation ability. However, ARC-overexpressing BMSCs showed a significant therapeutic effect on BRONJ by promoting osteogenesis and inhibiting osteoclasts. The BRONJ tissue treated with ARC-overexpressing BMSCs also showed a decreased level of cell apoptosis. Further the RNA sequencing and bioinformatics results suggested that ARC can regulate BMSCs by inhibiting the TNF-α (tumor necrosis factor-α) pathway increased in the BRONJ samples and may alleviate the disease by reducing pro-inflammatory potential of BRONJ-derived BMSCs. In summary, ARC-overexpressing BMSCs can effectively repair BRONJ necrotic bone tissue which provides new ideas for the clinical treatment of BRONJ.

Keywords: bisphosphonate-related osteonecrosis of the jaw; apoptosis repressor with caspase recruitment domain; bone marrow stem cells; stem cell transplantation; bone microenvironment

1. Background

Bisphosphonate-related osteonecrosis of the jaw (BRONJ) is a serious adverse reaction of bisphosphonate (BPs) application, which is a widely used inhibitor of bone resorption in patients suffered from bone metastases, osteoporosis, etc. BRONJ with clinical manifestations of exposed bone or sequestra located exclusively to the jaws over eight weeks [1,2], often leading to severe osteonecrosis, purulence, and fracture of the jaw bone, which is challenging to cure clinically and easy to relapse. Current clinical treatments are limited to pain-relieving and inflammation-controlling aimed at relieving BRONJ symptoms temporarily. The treatment eventually resorts to extensive resection of the jaw bone to restrict BRONJ from progressing further at the expenses of increasing living burden and impaired physical and mental health of patients [3]. Due to a lack of effective measures for etiological factors, long-term therapeutic effects are unsatisfying with a high risk of relapse [4,5]. Therefore, an in-depth exploration of the pathogenesis of BRONJ boasts crucial clinical significance for novel prevention and treatment strategies.

Previous studies have found that the pathological process of BRONJ is mainly characterized by altered bone remodeling with impaired bone resorption of osteoclasts and bone formation of osteogenic cells. Osteoblasts were the most frequently studied cell types among BRONJ related researches in cell viability, proliferation, differentiation, and migration [6]. However, few studies on BRONJ focus on the dysfunction of bone marrow mesenchymal stem cells (BMSCs), which are the source of osteoblasts. Yunsheng Li et al. have confirmed that BMSCs played an important role in mucosal healing and bone reconstruction during the process of BRONJ [7], suggesting improving the function of BMSCs may be potential targets for BRONJ therapy. In previous studies, systematic mesenchymal stem cells (MSCs) transplantation has been widely used to effectively treat a variety of diseases, such as adipose-derived mesenchymal stem cells (ASCs), dental pulp stem cells (DPSCs) and, of course BMSCs [8–10]. Moreover, osteonecrosis can be alleviated by MSCs transplantation therapy, and BMSCs in particular show strong therapeutic potential [11–13].

Apoptosis repressor with caspase recruitment domain (ARC), also termed NOL3, is an anti-apoptotic gene inhibiting extrinsic and intrinsic apoptosis pathways. In our previous research, ARC effectively inhibited the toxic effect of zoledronic acid on human mandibular BMSCs. Furthermore, we recently found that ARC promoted BMSCs osteogenic differentiation and new bone formation by activating the FGF-2/PI3K/AKT signaling pathway [14]. We thus speculated that the ARC gene overexpressing BMSCs can improve BRONJ through reversing stem cell injury in BRONJ tissues and promoting tissue healing.

In this study, BMSCs from the BRONJ rat model were isolated and cultured, and the impaired proliferation and osteogenesis ability of BMSCs was verified. ARC-overexpressing MSCs were then established and co-cultured with BRONJ-derived BMSCs and RAW264.7 cells respectively to test the recovery of the latter's osteogenic and anti inflammatory capability in vitro. At last, we systematically transplanted ARC-overexpressing BMSCs into BRONJ rats to observe their effect on curing BRONJ, and explored the possible mechanism by RNA sequencing.

2. Methods

2.1. Generate the BRONJ Model, BMSCs Isolation, and Culture

All animal experiments were approved by the Animal Research Committee of the Ninth People's Hospital Affiliated to Shanghai Jiao Tong University School of Medicine. To generate the BRONJ rat model, rats (Sprague-Dawley, male) were injected with zoledronic acid (0.1 mg/kg) combined with Dexamethasone (5 mg/kg) three times a week intraperitoneally. After two weeks of injection, the mandibular molars were extracted. BMSCs transplantation in rats was performed through tail vein injection after tooth extraction. All the rats were sacrificed two weeks later and were used for further experiments.

According to previous research methods [15], BMSCs were isolated and cultured for subsequent experiments. First, the rats were killed by overdose injection of ketamine and cervical dislocation. Then use micro scissors to cut both ends of the femur, tibia and mandible after they were dissected aseptically, and the bone marrow was washed quickly and sufficiently with prepared DMEM (dulbecco's modified eagle medium) using injection syringe. The prepared DMEM consisted of 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA), 100 U/mL penicillin, 100 U/mL streptomycins and 200 U/mL heparin (Sigma, St Louis, MO, USA). The primary cells were resuspended with DMEM after centrifugating at 2000 rpm for 10 min and then cultured at 37 °C in a steady atmosphere of 5% CO_2. Do not move the cell culture dish until the cells were attached to the dish. The DMEM was refreshed every three days, and the cells were used for the following experiment in passage 2 or 3.

RAW264.7 cells were obtained from Procell Life Science &Technology Co., Ltd. (Wuhan, China). Cells were suspended in DMEM, which contained 10% fetal bovine serum 100 U/mL penicillin, 100 U/mL streptomycin, and the culture conditions were consistent with those of BMSCs.

2.2. Cell Proliferation Detection

Cell proliferation ability was detected using cell counting kit-8 (CCK-8, Daojindao, Kumamoto Prefecture, Japan). BMSCs were inoculated on 96-well plates at a density of 1.5×10^3 cells per well, and the CCK-8 assay should be started after BMSCs were adherent and uniformly distributed. To assess the proliferation ability of BMSCs, 10 μL CCK-8 reagent was mixed with 100 μL DMEM and then added into each well. After incubation at 37 °C for 2 h, the absorbance value was measured at 490 nm with a microplate meter (Bio-Tek, Winooski, VT, USA). The CCK-8 assay requires four consecutive days of testing to dynamically monitor cell proliferation.

2.3. RNA Extraction, and Quantitative Reverse Transcription PCR (RT-qPCR) Analysis of Gene Expression

Total cellular RNA of BMSCs was extracted with TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). After lysis for 15 min, chloroform was added and mixed with TRIzol to incubate for 10 min. Then the samples were centrifuged at 4 °C at 12000 rpm for 15 min to collect the supernatant. Followed by this, an equal volume of isopropanol was added to the supernatant, and similarly centrifuged. Finally, RNA was obtained by gradient ethanol washing and drying. Subsequently, RNA reverse transcriptions were performed using PrimeScript RT reagent kit (Takara Bio, Inc., Otsu, Japan) according to the protocol to acquire cDNA first. In the RT-qPCR reaction system, a 10 μL SYBR Premix Ex Taq kit (Takara Bio, Inc., Otsu, Japan) was used to amplify 1 μL cDNA (mixed with 8 μL distilled water and 0.5 μL each primer). RT-qPCR was performed on a Bio-Rad iQ5 real-time PCR system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and the expression level of target genes was normalized to β-actin based on the $2^{-\Delta\Delta}$Cq method. Primers were designed and synthesized commercially (Shenggong Co., Ltd., Shanghai, China), and the corresponding primer sequences are provided in Table 1.

Table 1. Sequences of the primers used for RT-qPCR reaction.

Genes	Forward Primer	Reverse Primer
Acta1	TGAAGTGTGACGTGGACATC	GGAGGAGCAATGATCTTGAT
Alp	TCAAGCCAAACACAAACAGC	GGAGCCACAATCCAGTCATT
Ocn	GCGAGACATCAAGGAGAAGC	CCAATAAAGGAAGGCTGGAA
Runx2	GAAGAGGAGGAGGAAGAAGAGG	TCCATAGCCCAGTGTTGTAGC

2.4. Alkaline Phosphatase (ALP) Activity Detection and Alizarin Red Staining (ARS)

The C-BMSC (BMSC isolated from the standard control group) and D-BMSC (BMSC isolated from the BRONJ rat) cells were seeded at a density of 2.5×10^4 cells per well on 24-well plates and were cultured for 4 and 7 days. For ALP staining, 24-well plates inoculated with BMSCs were washed with PBS (phosphate-buffered saline) twice and fixed with 4% paraformaldehyde at 4 °C for 15 min. After removing the fixative, using PBS to rinse BMSCs twice, and the corresponding operations were the same as the preceding ones. Then the BCIP/NBT solution was added into each well to make ALP stained with the bluish violet dye, and cultured for 30 min in the dark condition. All the procedures are performed according to the ALP staining kit (Beyotime Institute of Biotechnology, Shanghai, China) instructions.

ALP activity was detected by the BCA (Bicin-choninic Acid) protein assay kit (Beyotime Institute of Biotechnology, Shanghai, China). By normalization with total protein content, ALP levels are presented as a percentage.

ARS was performed after 21 days of cell culture. The cells were first washed twice and then fixed, in a process consistent with ALP staining. Subsequently, 40 mM alizarin red dye solution was added into each well, and cultured for 20 min at room temperature. The dye was desorbed with 10% cetylpyridinium chloride (Sigma, St Louis, MO, USA) for 1 h, then the solution was collected and evenly distributed in the 96-well plate. Finally, the concentration of alizarin red was determined by a spectrophotometer (Bio-Tek, Winooski, VT, USA) at 590 nm wavelength.

2.5. Lentiviral Packaging and BMSC Transduction

Human embryonic kidney 293T cells (HEK293T) were used for lentivirus packaging. HEK293T cells were acquired from Genechem Co., Ltd. (Shanghai, China), originally purchased from American Type Culture

Collection (ATCC, Manassas, VA, USA). The cells were cultured in 10 cm dishes for 2–3 days until the fusion rate reached 90–95%. The recombinant virus plasmid pLV-Nol3-EGFP encodes the full-length nol3. The control vector pLV-EGFP and the packaging plasmids (pLP1:pLP2:pLP/VSVG = 2:2:1) were co-transfected into HEK293T cells using Lipofectamine™ 2000 (all from GeneChem Co., Ltd., Shanghai, China), and the optimal MOI value of the virus is 10. After 48 h of transduction, the lentiviral particles contained in the supernatant of 293T cells were collected and concentrated through a 0.45 μm filter. The concentrated solutions were then added to cultured BMSCs with infections ranging from 0 to 100. After 72 h of transduction, the expression of EGFP was observed by fluorescence microscopy, and the transduction efficiency was detected by western blot.

2.6. Jaw Bone Sample Preparation and Micro-CT Scanning

Two weeks after tooth extraction, the rats were sacrificed by overdose of ketamine injection and cervical dislocation. The maxilla and mandible of rats were dissected and fixed with 4% paraformaldehyde in a shaking table at 4 °C for 24 h. Micro-CT (SkyScan 1176, Bruker, Billerica, MA, USA) was used to evaluate the microstructure of the jaw bone. The fixed jaw bone sample was dried and placed in a tube for scanning. The specific scanning parameters were as follows: an X-ray tube potential of 65 kV, a tube current of 0.45 mA, and a voxel resolution of 18 μm. After the Micro-CT scan was completed, the bone tissue was visualized and reconstructed using the CTvol program (SkyScan) and NRecon software (SkyScan, USA). Percent trabecular area (BV/TV), bone volume (BV), and trabecular thickness (Tb.Th) were analyzed in the extraction area as ROI.

2.7. Histological Evaluation, Cell Apoptosis Analysis, and Tartrate Resistant Acid Phosphatase (TRAP) Staining

The jaw bone tissue samples after Micro-CT scan were immersed in 10% EDTA (Ethylene Diamine Tetraacetic Acid) solution for decalcificationin at room temperature in a shaking table for 10 weeks, and the decalcification solution was changed once a week. After decalcification is complete, the jaw bone tissue became soft and flexible. The samples were then treated with a gradient ethanol solution (75%, 80%, 90%, 100%, 100%) for dehydration, xylene for transparention, and finally embedded in paraffin. Then the samples were cut with a microtome (Leica, Hamburg, Germany) into 5 μm thick sections and mounted on glass slides. The slides were dried at 65 °C for one hour for further analysis. A TUNEL kit (Cell Signaling Technology, Inc., Boston, MA, USA) was used to detect the cell apoptosis level. The green fluorescence observed under fluorescence microscope represented apoptotic cells, and the mean fluorescence intensity was analyzed statistically with ImageJ. The apoptotic protein caspase-3 was evaluated by immunohistochemistry. The stained paraffin sections were observed under under a light microscope (Olympus Corporation, Tokyo, Japan), and Image-Pro 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA) was utilized for histomorphological analysis.

For TRAP staining, the sections were covered with TRAP incubation solution and incubated in a wet box at 37 °C for 60 min. Rinse the sections under running water for 5 min and then dye the nucleus with hematoxylin for 3 min. The neutral balsam was used to seal the sections and after that the sections were observed and analyzed. All the operations followed the TRAP staining kit instructions (Solarbio, Beijing, China). Cells with more than three nuclei and purplish-red TRAP particles are considered osteoclasts, which number was counted via Image-Pro Plus 6.0 as previously described [16].

2.8. RNA Sequencing (RNA-Seq) and Bioinformatics

Total RNA of ARC-overexpressing BMSCs and control BMSCs were collected and converted into cDNA for bioinformatics analysis. Based on R's DEGseq software package, we used MARS (MA-plot-based method with Random Sampling model) to analyze the overall gene expression level and identify significant differentially expressed genes between the two groups. Then, the functional analysis was performed using DAVID (v6.8) online tools and the result of KEGG pathway enrichment analysis was presented by bubble diagram. The number of enriched genes ≥ 2 and the significance threshold of hypergeometric test $p < 0.05$ were taken as significant enrichment results [14].

2.9. ELISA Analysis of TNF-α Concentration

TNF-α concentration of various samples were measured by the enzyme-linked immunosorbent assay (ELISA) technique using a rat anti-TNF-α antibody assay kit (Chondrex Inc., Redmond, WA, USA). To detect the TNF-α secretion of BMSCs, cells were incubated in 24-well plates at 37 °C with a serum-free medium. After corresponding treatment, we carefully collected the medium from the plates and stored at −80 °C for further analysis. Under sterile conditions, venous blood samples were harvested from the blood collection tube of rats,

and the serum was obtained by rapid centrifugation and the serum was quickly frozen at −80 °C. For ELISA analysis, 100 μL of the sample and standard solution were added to the 96-well plate respectively and incubated for two hours at 37 °C first. Then the plate was washed with specific washing buffer for three times, and drained on absorbent paper. TNF-α antiserum was added and incubated at 37 °C for one hour, followed by 100 μL streptavidin horseradish peroxidase reagent adding into the plate and the plate was incubated at 37 °C for another half an hour. After same washing procedures, 100 μL substrate solution (the ratio of TMB solution/hydrogen peroxide solution is 1:1) was added and incubated for 15 min in the dark condition at 37 °C. Finally, the stop solution was added to terminate the reaction. The spectrophotometer (Bio-Tek, Winooski, VT, USA) was used to measure the concentration of TNF-α at 450 nm. The standard curve was drawn and the concentration of TNF-α in samples was calculated.

2.10. Immunofluorescence Staining of RAW264.7 Cells

After co-culturing with the supernatant of BMSC-NOL3 and BMSC-CON for cell polarization analysis, the RAW264.7 cells were fixed in 4% paraformaldehyde for 30 min at room temperature and permeabilized with 0.25% Triton-X and then blocked by 1% BSA (Bovine Serum Albumin). The primary antibodies against iNOS (inducible nitric oxide sythase), CD163 and MHC II (major histocompatibility complex II) were used to incubated with cells overnight at 4 °C. Then the RAW264.7 cells were incubated with tetramethylrhodamine isothiocyanate-conjugated secondary antibody for 30 min. The nuclei were stained with DAPI for 15 min. Fluorescent images were boserved and obtained through an inverted fluorescence microscope (CKX53, Olympus, Tokyo, Japan). The corresponding analysis was carried out through the MShot Image analysis system of the microscope.

2.11. Statistic Analysis

Statistical analysis was conducted using GraphPad Prism 6 software package. Data were expressed as means ± standard deviation (SD). Differences between the two groups are analyzed by independent sample t-test, while a one-way ANOVA is used to test multiple groups. Statistically significant difference were considered at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

3. Results

3.1. Combination of Bisphosphonate and Dexamethasone Treatment Induced BRONJ Disease in Rats

First, we built the BRONJ rat model through zoledronic acid and dexamethasone injection combined with tooth extraction (Supplementary Figure S1A). The rats that successfully established BRONJ disease model showed incomplete healing of mucosa and alveolar bone exposure (Supplementary Figure S1B), and the Micro CT revealed an obvious necrotic bone area at tooth extraction socket (Supplementary Figure S1C), consistent with the clinical symptoms of BRONJ. These BRONJ rats were prepared for the following experiments.

3.2. BRONJ-Derived BMSCs Exhibited Decreased Proliferation Ability and Impaired Osteogenic Differentiation Ability

We isolated and cultured bone marrow mesenchymal stem cells from the BRONJ rats (D-BMSC) to assess its proliferation and osteogenic ability. As expected, the D-BMSC showed reduced cell proliferation potential than the C-BMSC (BMSCs isolated from control rats) (Figure 1c). RT-qPCR results of specific osteogenic genes showed consistent trend regardless of osteogenic inducing time between the two group. Both of the early-stage osteogenic marker (*Alp*) and the later-stage osteogenic marker (*Ocn*, osteocalcin), as well as an essential factor in regulating the bone formation (*Runx2*, runt-related transcription factor 2) were downregulated in the D-BMSC group, (Figure 1a). Meanwhile the C-BMSC group yielded stronger ALP staining on day 4 and day 7, indicating the osteogenesis of the D-BMSC group was less than the C-BMSC group (Figure 1b,d). ARS results revealed that less calcium deposition was formed in the D-BMSC group on day 21, consistent with ALP staining (Figure 1e,f). These results confirmed poor proliferation and osteogenic ability of BRONJ-derived BMSCs, underling the necessary improvement of BMSCs' capacity in BRONJ.

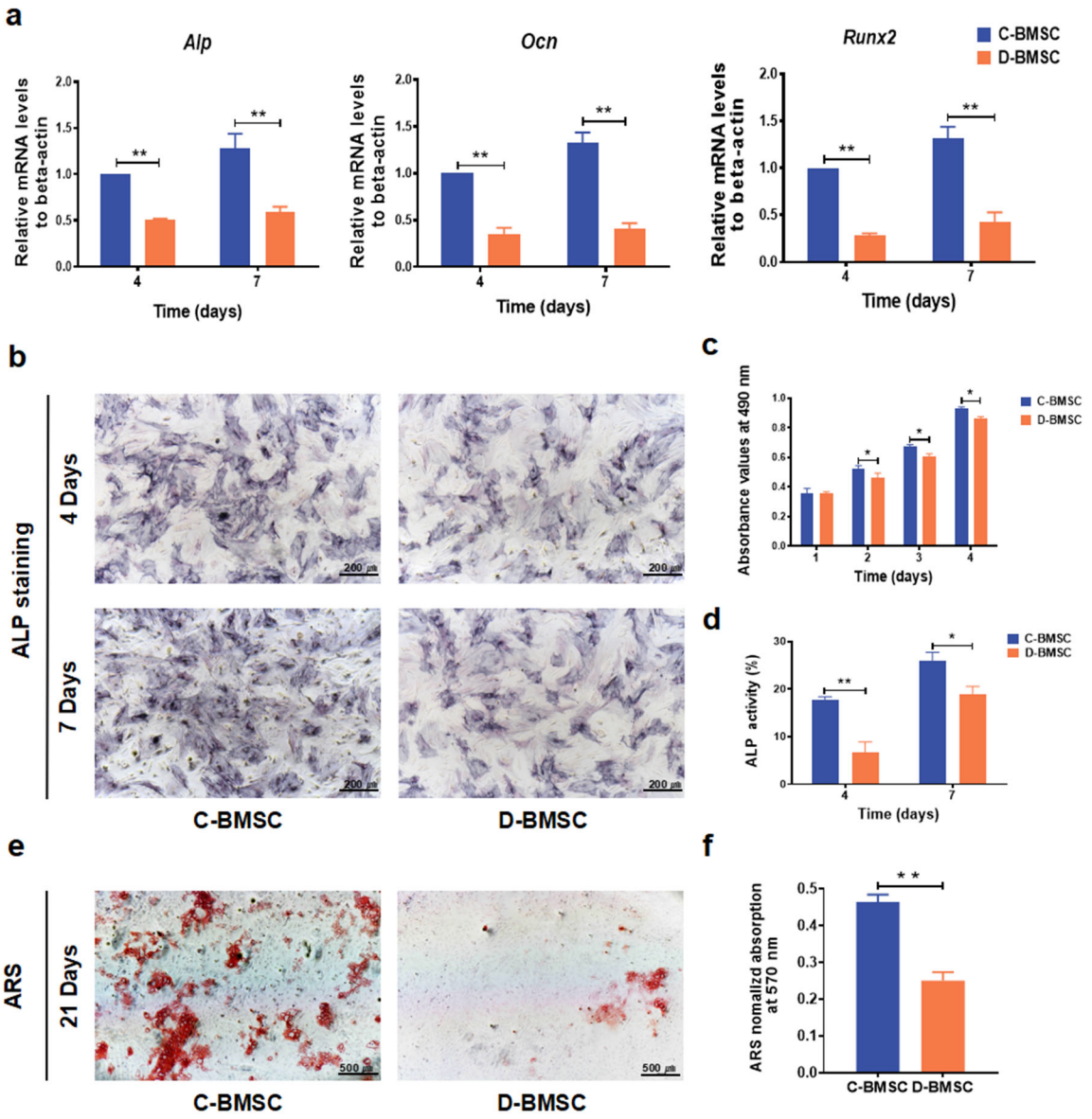

Figure 1. BRONJ-derived BMSCs showed decreased proliferation ability and impaired osteogenic differentiation ability. (**a**) RT-qPCR analysis of osteogenic genes expression level in both C-BMSC and D-BMSC groups, including *Alp*, *Ocn*, and *Runx2* (n = 3 rats, ** $p < 0.01$). (**b**) ALP staining of C-BMSC (left column) and D-BMSC (right column) on day 4 and 7. (**c**) Cell proliferation detection by the CCK-8 method from day 1 to day 4 in each group (*$p < 0.05$). (**d**) Semiquantitative analysis of ALP activity (* $p < 0.05$, ** $p < 0.01$). (**e**) ARS results of C-BMSC (left column) and D-BMSC (right column) on day 21. (**f**) Semiquantitative analysis of ARS (* $p < 0.05$, ** $p < 0.01$).

3.3. ARC-Overexpressing BMSCs Showed Significant Therapeutic Effect on BRONJ by Promoting Osteogenesis and Inhibiting Bone Resorption

Since our previous studies have shown that ARC was capable of inhibiting the apoptosis of BMSCs and increasing bone formation, we established ARC-overexpressing BMSCs (NOL3-OE) using a lentivirus transduction method to explore its potential for treating BRONJ. Three days after lentivirus transduction, cells were observed under a light and fluorescence microscope, and more than 70% BMSCs were EGFP-positive, suggesting a high transduction rate (Figure 2a). The successful construction of ARC-overexpressing BMSCs was further validated by ARC protein expression level in the transfected BMSCs and it was significantly up-regulated compared with the vector control cells (EV control) (Figure 2b). Then we evaluated the therapeutic potential of ARC-overexpressing BMSCs in vitro first. The D-BMSC co-cultured with the supernatant of ARC-overexpressing BMSCs showed obvious recovery of osteogenic ability detected by ALP staining and ARS (Figure 2c–f). The expression level of osteogenic genes including *Alp*, *Ocn*, and *Runx2* were increased dramatically after co-culturing

with the supernatant of ARC-overexpressing BMSCs as shown in Figure 2g. These results strongly suggested that ARC-overexpressing BMSCs improved the impaired osteogenic ability of BMSCs derived from BRONJ, indicating it may be promising for the treatment of BRONJ.

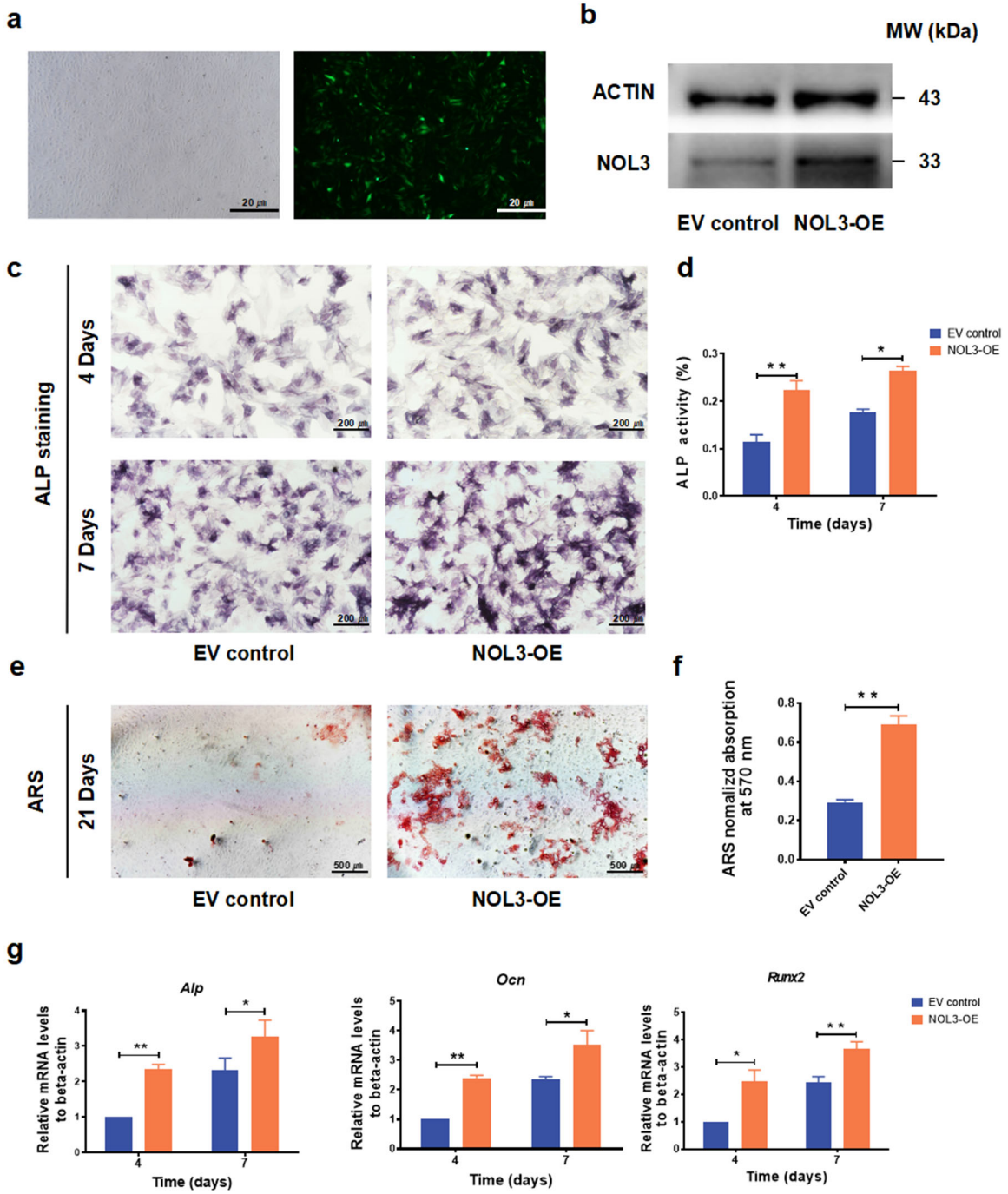

Figure 2. D-BMSCs co-cultured with the supernatant of ARC-overexpressing BMSCs showed recovery of osteogenic ability. (**a**) EGFP expression in BMSCs under fluorescence microscopy after lentiviral infection. (**b**) ARC protein expression detected by western blot in the transfected BMSCs. (**c**) ALP staining of D-BMSCs co-cultured with the supernatant of control BMSCs (**left column**) and ARC-overexpressing BMSCs (**right column**) on days 4 and 7 (* $p < 0.05$, ** $p < 0.01$). (**d**) Semiquantitative analysis of ALP activity (* $p < 0.05$, ** $p < 0.01$). (**e**) ARS results on day 21. (**f**) Semiquantitative analysis of ARS (** $p < 0.01$). (**g**) RT-qPCR analysis of osteogenic genes expression level of each group, including *Alp*, *Ocn*, and *Runx2* (* $p < 0.05$, ** $p < 0.01$).

Then, to directly assess the possible therapeutic effect of ARC-overexpressing BMSCs on BRONJ tissue, ARC-overexpressing BMSCs were infused into BRONJ rats by vein tail infusion (Figure 3a). Two weeks after tooth extraction, in BRONJ-CON (BRONJ rats infused with EV control BMSCs) and BRONJ-NOL3 (BRONJ rats infused with NOL3-OE BMSCs) groups, the open wound of oral mucosa generally closed over the tooth

extraction site. Micro-CT was performed to evaluate new bone formation in the tooth extraction site in both groups (Figure 3b). As shown in Figure 3c, BRONJ-NOL3 group represented a significantly increase number of BV, BV/TV and Tb.Th compared with the BRONJ-CON group, suggesting the bone formation process was more active. (The bone volume (BV) value was 0.1367 ± 0.007823 in the BRONJ-CON group, while that of BRONJ-NOL 3 group was 0.2033 ± 0.008122. The value of BV/TV was 22.37% ± 1.468% in the BRONJ-CON group, and 32.53% ± 1.538% in the BRONJ-NOL3 group. The value of Tb.Th was 0.1167 ± 0.008819 μm in the BRONJ-CON group, and 0.1933 ± 0.01453 μm in the BRONJ-NOL3 group). These data confirmed that ARC did play a vital role in BMSC-mediated new bone regeneration.

Then we also evaluated bone resorption activity in the tooth extraction area. TRAP staining of the tissue section demonstrated less TRAP positive osteoclasts in the BRONJ-NOL3 group (Figure 3d). The number of osteoclasts was 16.17 ± 0.7032 in the BRONJ-CON group, while this number was reduced by nearly two-thirds to 6.667 ± 0.7601 in the BRONJ-NOL3 group per area (Figure 3e), indicating that the ARC-overexpressing BMSCs effectively inhibited the osteoclast activity of BRONJ tissues.

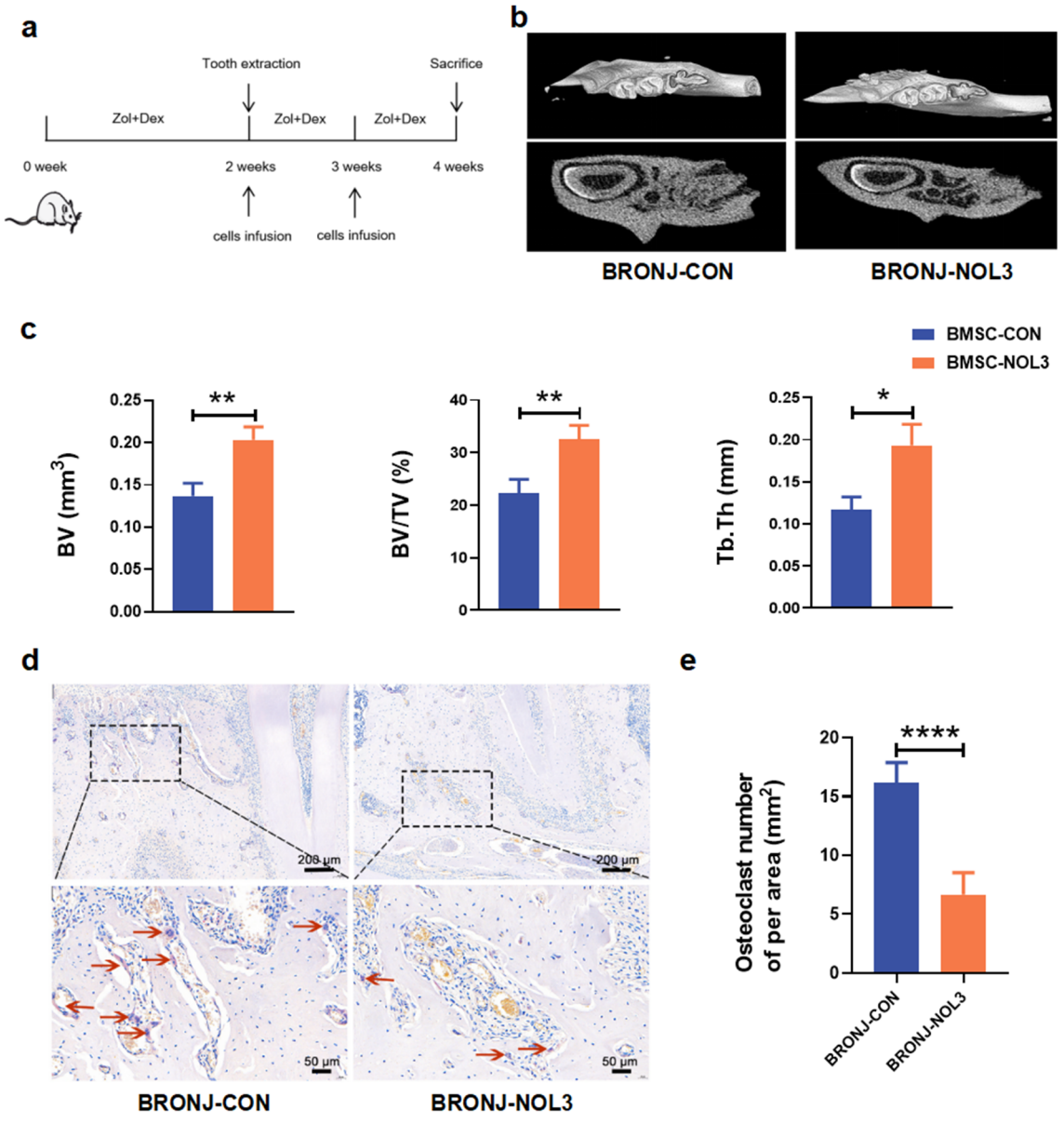

Figure 3. ARC-overexpressing BMSCs exerted significant therapeutic effect on BRONJ in vivo. (**a**) Experimental protocol for ARC-overexpressing BMSCs transplantation in BRONJ rats. The rat received both Zol and Dex administered intravenously for two weeks with tooth extraction. Cells were infused twice at 0 and one week after tooth extraction (n = 3 rats for each group). (**b**) Micro-CT scan of the tooth extraction site in BRONJ-NOL3 and BRONJ-CON groups. (**c**) Mean values and standard deviation of BV, BV/TV, and Tb.Th in both groups. (* $p <$ 0.05, ** $p < 0.01$). (**d**) TRAP staining revealed less osteoclast numbers in the BRONJ-NOL3 group (the red arrow points to osteoclasts). (**e**) Osteoclasts number per area in each group (**** $p < 0.0001$).

3.4. ARC-Overexpressing BMSCs Inhibits Apoptosis in the BRONJ Tissue

As ARC is an anti-apoptotic gene inhibiting extrinsic and intrinsic apoptosis pathways, inhibition of apoptosis may be an important way for ARC-overexpressing BMSCs to treat BRONJ. Therefore, we investigated the influence of ARC-overexpressing BMSCs on the apoptosis of the BRONJ tissue. As expected, immunohistochemistry analysis showed the apoptosis related protein caspase-3 was decreased in the BRONJ-NOL3 group than in BRONJ-CON group (Figure 4a), consistent with the statistical analysis of the expression of Caspase3 (Figure 4c). Moreover, cell apoptosis activity was greatly reduced in the BRONJ-NOL3 group observed by a TUNEL kit (Figure 4b,d), demonstrating the inhibitory effect of ARC-overexpressing BMSCs on apoptosis in BRONJ.

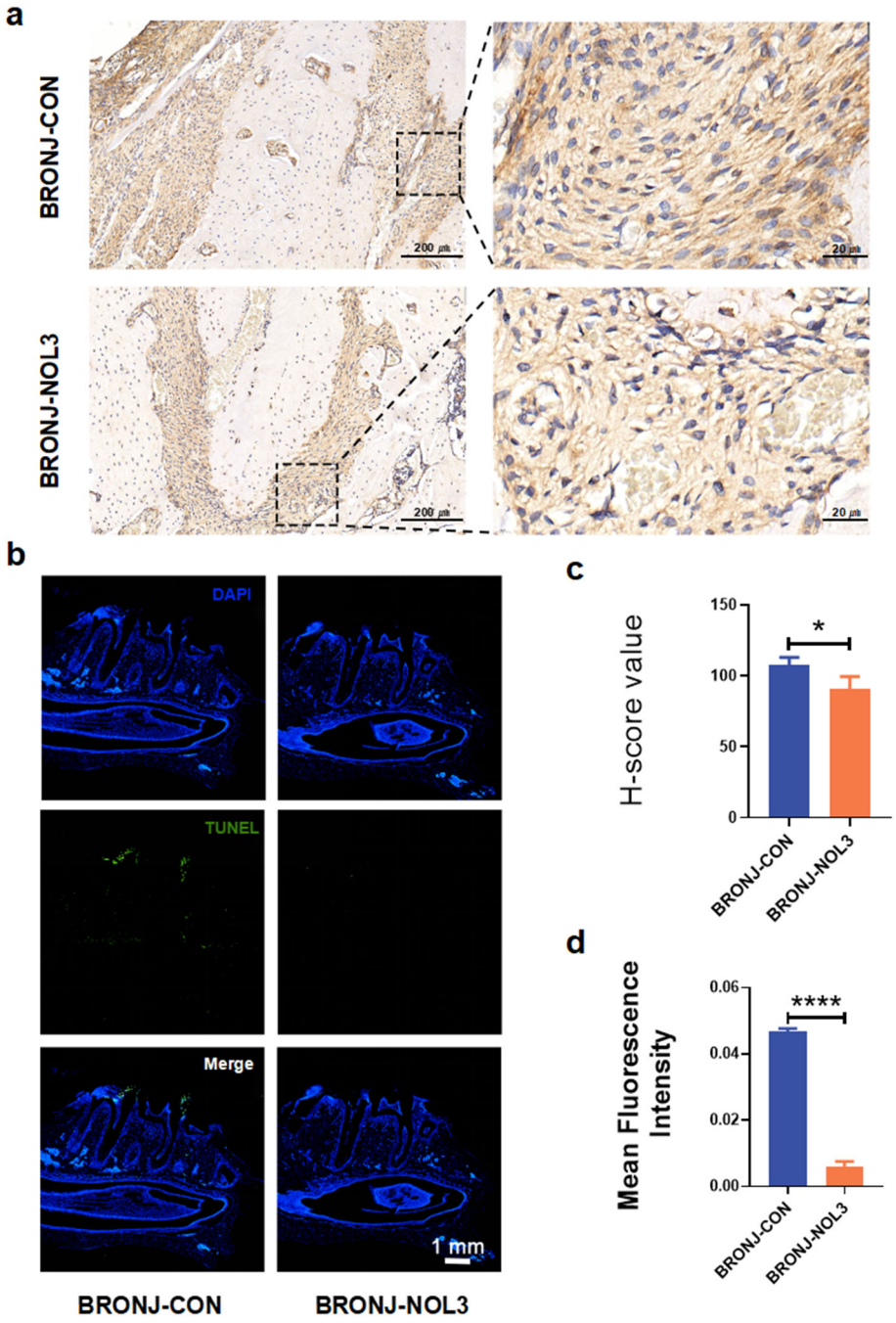

Figure 4. ARC-overexpressing BMSCs inhibited cell apoptosis in vivo. (**a**) Immunohistochemistry analysis of caspase-3 of the tooth extraction site in BRONJ-NOL3 and BRONJ-CON groups. (**b**) Immunofluorescence staining was carried out to detect cell apoptosis. Nuclei were stained with DAPI in blue. Cell apoptosis was detected by a TUNEL kit and stained green. (**c**) Analysis of H-score value of both groups in (**a**) (* $p < 0.05$). (**d**) Mean Fluorescence Intensity of both groups in (**b**) (**** $p < 0.0001$).

3.5. ARC May Promote the Cure of BRONJ by Increasing the Anti-Inflammatory Potential of D-BMSC

To further explore the possible mechanism of ARC-overexpressing BMSCs promoting bone formation, we then conducted RNA-seq of EV control and NOL3-OE BMSCs. The bioinformatics results identified that TNF signaling pathway and osteoclast differentiation pathway were both inhibited in NOL3-OE BMSCs (Figure 5a). This was consistent with the fact that ARC is an inhibitor of the TNF-α/NF-κB signal pathway.

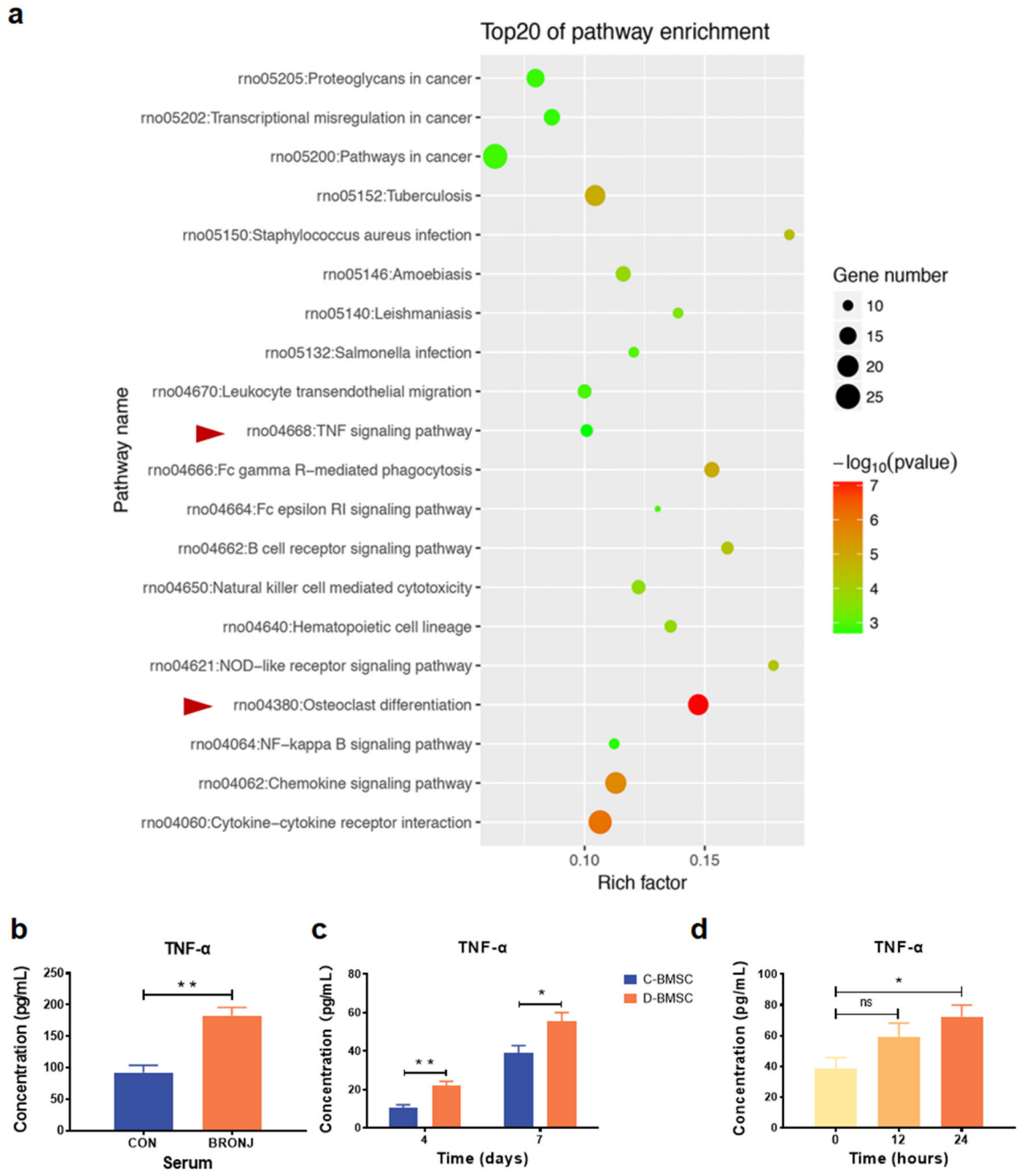

Figure 5. ARC may promote the cure of BRONJ by increasing the anti-inflammatory potential of D-BMSCs. (**a**) KEGG pathway enrichment analysis. Pathway enrichment of differentially expressed genes between ARC-overexpressing BMSCs and control BMSCs. y-Axis indicates the pathway name, x-axis indicates the enriched factor in each of pathways. The bubble size indicates the number of genes. The color bar indicates the corrected p-value, the blue represents higher value, the red represents lower value. (**b**) TNF-α concentration of serum tissue from BRONJ and control rats was detected by ELISA kit (n = 3 rats for each group, $^{**}p < 0.01$). (**c**) TNF-α concentration of BMSCs supernatant from BRONJ and control rats ($^{*}p < 0.05$, $^{**}p < 0.01$). (**d**) TNF-α concentration of BMSCs supernatant after treatment with zoledronic acid for different hours ($^{*}p < 0.05$).

Previous study has proven that BMSCs played an important role in immune regulation of bone marrow microenvironment and may be involved in promoting the chronic inflammatory environment in necrotic jaw tissue

characterized by the enhancement of inflammatory cytokines [17]. In consideration of the TNF-α signaling pathway in BMSCs was analyzed as the key regulatory target of ARC in our bioinformatics results, we speculated that the expression of TNF-α may be abnormally activated in D-BMSC and damaged the tissue microenvironment. To verify our hypothesis, different tissue and cell samples were collected to evaluate the expression of TNF-α. The concentration of TNF-α was 182.5 ± 6.561 pg/mL in the serum of BRONJ rats which was twice the concentration of the control group (Figure 5b). Accordingly, the supernatant concentration of TNF-α was 10.42 ± 0.9061 pg/mL in the C-BMSC group and 22.06 ± 1.263 pg/mL in the D-BMSC group on day 4, 39.07 ± 2.164 pg/mL in the C-BMSC group, and 55.64 ± 2.518 pg/mL in the D-BMSC group on day 7 (Figure 5c). Moreover, the BMSCs supernatant concentration of TNF-α after treating with 20 μM zoledronic acid was 38.13 ± 4.365 pg/mL at 0 h, 59.32 ± 5.100 pg/mL at 12 h, and 72.40 ± 4.359 pg/mL at 24 h, no statistical difference between 12 h and 0 h while significant higher at 24 h than 0 h according to Figure 5d. These data indicated that the secretion level of TNF-α was significantly higher in the BRONJ rats and tissues. Since TNF-α is a classic pro-inflammatory factor and may significantly affect the immune process, suggesting variational immune microenvironment may occur through TNF-α augment in BRONJ.

Thus the supernatant of EV control and NOL3-OE BMSCs were collected and then co-cultured with RAW264.7 cells to evaluate the effect of ARC on macrophage polarization. The results showed that the number of M1 macrophages decreased significantly after co-culturing with the supernatant of ARC-overexpressing BMSCs (Figure 6a), while M2 macrophages increased markedly (Figure 6b), suggesting that ARC exerted strong anti-inflammatory potential. These results suggested that ARC may ameliorate BRONJ through enhancing the anti-inflammatory ability of BMSCs.

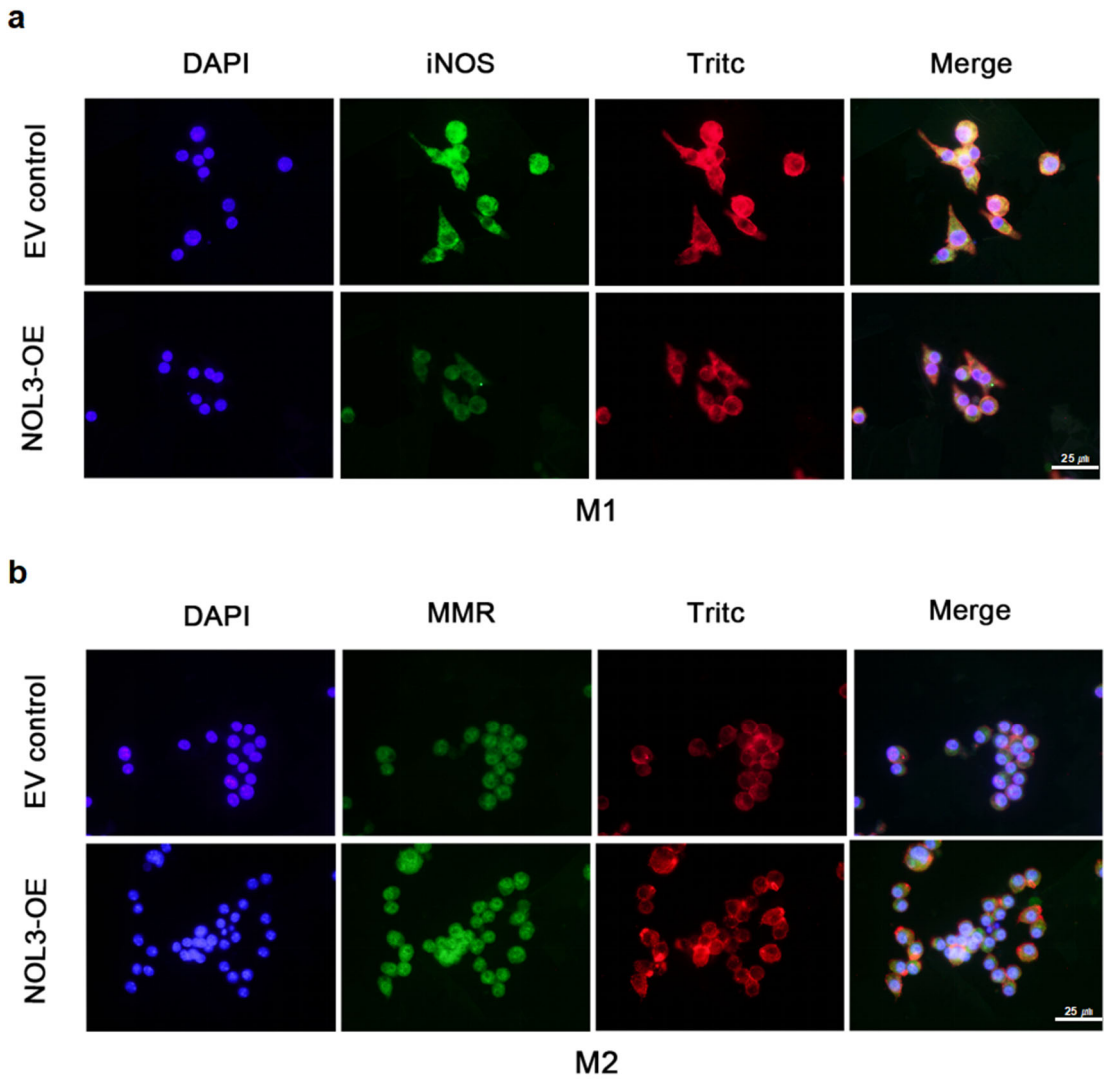

Figure 6. ARC inhibited M1 macrophage polarization but increased M2 phenotype polarization. (**a**,**b**) Representative immunofluorescent images of M1 and M2 macrophages.

4. Discussion

Medication-related osteonecrosis of the jaw results from multiple factors, including the impairment of bone remodeling, the limitation of bone vascularization, and immune dysfunction. BMSCs are multi-functional stem cells that can promote bone regeneration, facilitate bone angiogenesis and regulate immune function. Several studies have shown that BMSCs damage plays a crucial role in the occurrence of BRONJ [18]. In this study, the osteogenic differentiation and proliferation of BRONJ jaw-derived BMSCs were detected in vitro and in in vivo. We found that the osteogenic differentiation and proliferation of BRONJ jaw-derived BMSC were decreased, further confirming that the function of BRONJ jaw-derived BMSCs was impaired. Therefore, targeting improving the osteogenic ability of BMSCs in BRONJ may become a prospective treatment.

BMSCs have many applications in various fields of regenerative medicine. They have already been proven to offer effective therapeutic modalities in preventing the development of BRONJ. Although BMSCs possess anti-inflammatory and reparative functions, they are not always sufficient to achieve the best results in stimulating regeneration. In this regard, we genetically modified MSCs to enhance their therapeutic action towards BRONJ.

ARC is a powerful multifunctional anti-apoptotic agent, which plays a crucial role in inhibiting cell apoptosis and cell death and exhibits an obvious reparative function in damaged tissues. In our previously published research, we found that ARC can effectively inhibit the toxic effect of zoledronic acid on human osteoblasts [19]. We also found that ARC can effectively promote BMSCs osteogenic differentiation and new bone formation by activating the Fgf-2/Pi3k/Akt signaling pathway [14], indicating its powerful potential in curing BMSCs disorder in BRONJ. In this research, we established ARC-overexpressing BMSCs to comprehensively evaluate its therapeutic effect on BRONJ jaw-derived BMSCs in vitro, as well as BRONJ rats in vivo. The results showed that ARC-overexpressing BMSCs could effectively promote the osteogenic differentiation of BRONJ jaw-derived BMSCs. Identified with the in vitro experiment, after infusing ARC-overexpressing BMSCs into BRONJ rats, more new bone formation were observed in the tooth extraction site. We then confirmed that ARC could inhibit cell apoptosis of the BRONJ tissue. In addition to the apoptosis, we also evaluated the effect of ARC on osteoclast activity, as osteoclast turbulence plays a critical role in the occurrence and development of BRONJ. Fewer osteoclasts existed in the BRONJ-NOL3 group, indicating reduced bone resorption in BRONJ rats receiving ARC-overexpressing BMSCs. All the above results confirmed that ARC-overexpressing BMSCs showed significant therapeutic effect on BRONJ by promoting osteogenesis while inhibiting osteoclast activity and cell apoptosis.

Meanwhile, RNA-seq and bioinformatics were conducted to reveal the important mediators of ARC's effect in the molecular level. The enrichment results of the KEGG pathway analysis of differentially expressed genes showed that TNF-α and NF-κB signaling pathway were inhibited in ARC-overexpressing BMSCs. Interestingly, the KEGG pathway analysis also revealed osteoclast differentiation signaling pathway was suppressed, which further confirmed that transplantation of ARC-overexpressing BMSCs can inhibit the osteoclast activity in BRONJ tissues.

MSCs can modulate the function and phenotype of immune cells in bone, such as BMSCs and periodontal ligament stem cell (PDLSC) [20,21], and are proven to enable immune cells' phenotypic conversion into anti-inflammatory and immunosuppressive cells. Haploidentical mesenchymal stem cells displaying immunosuppressive effects on certain conditions have an immune-regulatory capacity and thus can cure inflammatory diseases [22]. In this study, the inhibition of TNF-α pathway in ARC-overexpressing BMSCs suggested that TNF-α may play an important role in impairing the immune regulation function of BRONJ jaw-derived BMSCs and promoting the inflammatory disease. Therefore, combined with RNA-Seq results, we hypothesized that ARC-overexpressing BMSCs transplantation may involve in modulating immune processes in the bone microenvironment to improve BRONJ. As expected, we systematically detected the higher levels of TNF-α in BRONJ samples, including the expression of TNF-α in the supernatant of BMSCs derived from BRONJ jaws, blood samples of BRONJ rats, and the expression of TNF-α in BMSCs after zoledronic acid treatment. These results confirmed that the expression of TNF-α was abnormally elevated in BRONJ samples and especially in BMSCs. As TNF-α is a classical proinflammatory cytokine, thus we further detected the anti-inflammatory ability of ARC by co-culturing the supernatant of EV control and NOL3-OE BMSCs with RAW264.7. The results showed the anti-inflammatory capacity of NOL3-OE BMSCs by facilitating M2 macrophage polarization. From this, we speculated that functional impairment of BRONJ jaw-derived BMSCs may be partly due to the abnormal activation of TNF-α signaling pathway, while ARC-overexpressing BMSCs partially recovered the anti-inflammatory function and treated BRONJ effectively, but further studies on its specific molecular pathways are still needed.

5. Conclusions

Based on the above research results, we found that ARC overexpressed BMSCs can effectively repair BRONJ necrotic bone tissue, and this therapeutic effect may achieved by ARC through regulating the levels of cell apoptosis and inflammation in BRONJ tissues. However, this study still has some limitations, we will further explore the possible molecular mechanism for ARC to improve BRONJ jaw-derived BMSCs function and the molecular regulation mechanism of TNF-α on the immune regulatory function of these stem cells, so as to promote the clinical use of ARC overexpressed BMSCs in the treatment of bone-related diseases such as BRONJ.

Supplementary Materials

The following supporting information can be downloaded at: https://www.sciltp.com/journals/rmd/2024/1/559/s1, Figure S1: Combination of bisphosphonate and dexamethasone treatment enhanced BRONJ disease in rats. (A) Experimental protocol for generating the BRONJ model. The rats received both Zol and Dex administered intravenously for two weeks with tooth extraction. (B) The open wound of oral mucosa over the extraction site in BRONJ rats. (C) Micro-CT scan of the extraction site in BRONJ rats.

Author Contributions

R.J. performed the cell experiments, and was a major contributor in writing the manuscript; Y.D. played a significant role in creating animal models and the acquisition of the data; Y.Z. and J.W. involved in the data processing; X.J. conceived the study and critically revised the manuscript; L.H. designed the work, supervised the study and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Animal Research Committee of the Ninth People's Hospital Affiliated to Shanghai Jiao Tong University School of Medicine (the document code is SH9H-2020-TK277-1 and the date of approval is 12 March 2020).

Data Availability Statement

All data generated or analysed during this study are included in this published article.

Conflicts of Interest

The authors declare no competing financial interests.

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