ORIGINAL RESEARCH

Ruan Jian Qing Mai's Formula Promotes Bone Marrow-Derived Mesenchymal Stem Cell Migration and Proliferation

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ABSTRACT

Objective • To investigate the response of (BM-MSCs) to the Ruan Jian Qing Mai formula (RJQM) in the treatment of atherosclerotic occlusion (ASO), and consequently promoting the development of collateral circulation and angiogenesis.

Method • 35 male rats were randomly assigned to 6 experimental groups and A control group. 0.9% NaCl solution and 2.7, 5.4, 10.8, 16.2, 21.6, and 27 $g \times kg^{-1} \times d^{-1}$ of RJQM formula were gavaged to the experimental groups twice a day for 8 days. After the last administration, medicated serum was prepared from the blood collected from the abdominal aorta. The human BM-MSCs were divided into an experimental group and a control group. A blank group of cells was added with a complete medium without rat serum; an experimental group of cells was added with the prepared drug-containing serum. Under hypoxic conditions, the drug-containing serum was used to treat BM-MSCs and/or endothelial cells of human umbilical vein (HUVECs). A Cell counting kit (CCK8) was used to detect cell proliferation. Western blot (WB) and quantitative real-time PCR (qPCR) were used to identify related genes expression.

Results • The results of this study showed that the purity of the BM-MSCs was >95%. The drug-containing serum significantly rise in CCND1 expression (encoding cyclin D1) and *MYC*, especially when the concentration of medicated serum was 10.8 $g \times kg^{-1} \times d^{-1}$. Treatment of either BM-MSCs or HUVECs alone or both with medicated serum aids in the spread of mesenchymal stem cells from the bone marrow to HUVECs. qPCR results showed that the mRNA expression of CCL2, CCL3, CCL25, IL8, IGF1, and PDGFB increased dramatically after treatment with medicated serum. The expression of the corresponding receptors for these up-regulated chemokines was detected in BM-MSCs, and it was found that CXCR1, CXCR4, CXCR7, and PDGFRB were up-regulated.

Conclusion • This study provides a preliminary understanding of the mechanism of RJQM in the treatment of ASO. (*Altern Ther Health Med.* 2023;29(8):172-177).

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INTRODUCTION

Arteriosclerosis obliterans (ASO) is a major subtype of peripheral artery diseases (PADs).[1] Epidemiologically, there are more than 200 million PAD patients worldwide. which ~~has~~ increased by 20% in the past decade.[2,3] The causes of ASO are multifactorial including, dyslipidemia, diabetes mellitus (DM), cigarette smoking, and hypertension. Patients with PAD often manifest severe comorbidities including, respiratory dysfunction, end-stage renal disease (ESRD) necessitating dialysis, coronary artery disease (CAD), and chronic heart failure (CHF), leading to CHF.[4] PAD end-stage syndrome, also known as critical limb ischemia (CLI), poses a significant risk of losing the lower limb. Furthermore, CLI is also strongly associated with cardiovascular events, including myocardial infarction and death.[5]

For ASO patients, arterial revascularization is the most imperative procedure for salvaging critical limb ischemia. The

most effective way to achieve revascularization is bypass surgery.[6] Due to the poor systematic conditions of ASO patients and the frequent comorbidities highly invasive surgeries are difficult to perform, leaving limited treatment options. After more than 50 years of clinical practice and scientific research, Professor Jiuyi Xi, an expert on vascular disease in traditional Chinese medicine, proposed the treatment of ASO by the Ruan Jian Qing Mai formula (RJQM). RJQM, a multi-component herbal formula, has been widely used to treat peripheral arterial disease (PAD) in China. However, its active compounds and mechanisms of action are still unknown. Preliminary clinical trials in the TCM-Integrated hospital found that RJQM significantly improved the hemoglobin and blood oxygen saturation of the lower limb of stages I, II, and III ASO patients better than other traditional Chinese medicine groups, such as Huoxue Huayu Decoction (Danshen Extract Tablets). Moreover, several clinical studies have shown that using the RJQM formula has lipid level-lowering effects, protects the endothelium cells, improves vasomotor function, establishes collateral circulation, and improves limb blood flow,[7] However, the mechanism is still unclear.

Multipotent mesenchymal stem cells (MSCs) are a subtype of stem cells that can self-renew and differentiate. MSCs can be extracted from other tissues other than the bone marrow, such as the liver, adipose, muscle, placenta, umbilical cord blood, and skin.[8] According to the culturing conditions, MSCs can differentiate into several cell types, including endothelium, osteoblast, chondrocyte, and adipocytes.[9,10] Recently, MSCs are considered therapeutic agents for skin regeneration and rejuvenation. MSCs can contribute to immunological and inflammatory responses following BM-MSCs-induced T-cell suppression.[11] In addition, MSCs secrete large quantities of growth factors, cytokines, and chemokines and have a role in the stimulation of metabolism. These growth factors are crucial for immunomodulatory processes, such as controlling the implantation of hematopoietic stem cells, their development, and the control of apoptosis and angiogenesis.[12] MSCs are frequently employed in treating a wide range of diseases, including cardiovascular diseases, blood system disorders, nervous system diseases, immune control, and orthopedic diseases, due to their exceptional capabilities for multipotential differentiation.[13] In MSCs-mediated therapy, they can target and migrate to the injured site to repair the damaged tissue.[14] Therefore, we aim to explore whether MSCs can also repair tissue injury in treating ASO.

As far we know, this is the first study to investigate the response of bone marrow-derived mesenchymal stem cells (BM-MSCs) to the Ruan Jian Qing Mai formula (RJQM) in the treatment of atherosclerotic occlusion (ASO), consequently promoting the development of collateral circulation and angiogenesis.

MATERIALS AND METHODS

Preparation of Ruan Jian Qing Mai formula

The Chinese medicines needed for the preparation of RJQM formula were provided by the Pharmacy Department of Shanghai TCM-Integrated Hospital including 30 g of weeping grass, 15 g of seaweed, 10 g of Siegesbeckia tenuis, 15 g of cattail yellow, and 30 g of calcined oyster, a total of 100 g. Conventional methods were prepared for the decoction.[15] These medicines are heated to a boil over high heat and simmer for 20 to 30 minutes. Then drain off the juice. Finally, it was concentrated to 1g/ml of crude medicine is obtained and then can be stored at 4°C after preheating in a water bath.

Preparation of drug-containing serum

Thirty-five male Sprague-Dawley (SD) rats, ten weeks, were randomly divided into six experimental groups and a control group. With 5 rats in each group.In the control group,they were given 0.9% NaCl solution and hey ,while in the other six experimental groups were given 0.9% NaCl solution and 2.7, 5.4, 10.8 (clinical equivalent), 16.2, 21.6 and 27 $g \times kg^{-1} \times d^{-1}$ RJQM formula respectively, twice a day, gavage for 8 days. After the last administration, medicated serum was prepared from the blood collected from the abdominal aorta. After standing for 1 hour, the collected blood was centrifuged at 2500 rpm for 10 minutes, the supernatant was pipetted, then it was filtered, sterilized, aliquoted, and stored at 4°C. All animal experiments were carried out after approval from Shanghai Jiao Tong University's ethical committee and were conducted accordingly to the committee's guidelines.

Isolation and culture of MSCs from human bone marrow tissue

Samples are collected by performing bone marrow explants on ASO patients' bone marrow. The ethics committee of Shanghai TCM-Integrated Hospital reviewed and approved this clinical trial (NO.2021-003-1). Using a previously published procedure, bone marrow was used for taking out the human BM-MSCs.[15] In a nutshell, 1×10^6 mononuclear cells from the bone marrow were injected into a 100 mm culture dish as a single-cell suspension. Adherent cells were cultured in DMEM (*Thermo Fisher Scientific, Waltham, MA,* 11965-118) with fetal bovine serum (5%) added as a supplement (*Thermo Fisher Scientific*), 100 μg/mL streptomycin and 100 U/mL penicillin (*all from Gibco/ Thermo Fisher Scientific, Waltham, MA, 15140122*). At 80% of confluence, MSCs developing as adherent cells were harvested and expanded.

Characterization of MSCs

The adhering cells from the third to fifth passages were isolated with 10 mM EDTA, washed with PBS, and stained with antibodies for FACS analysis to characterize BM-MSCs. 0.5 μL antibodies were added to the cell suspension, incubated for 15min at room temperature then washed with PBS, and centrifuged at 1500 rpm for 3min. The supernatant was discarded and then cells were resuspended with 350 μL PBS for analysis. The following panel of antibodies was used: anti-CD11b (APC-eFluor780), anti-CD29 (102215, Biolegend, APC), anti-CD34 (PE-Cy7), anti-CD44 (APC-eFluor780),

anti-CD45 (eFluor506) and anti-CD90 (PE-Cy7) (all from *BioLegend or eBioscience*). Data collection from 10 000 events per sample was recorded in BD LSRFortessa cell analyzer (*BD Biosciences, 2010118AA*), and analyses were performed using FlowJo software (*BD Biosciences*).

Cell proliferation assay

We quantified cell growth using a Cell Counting Kit-8 (CCK-8) (Dojindo, Japan, CK04-11) following the manufacturer's recommendations. Absorbance was determined using a microplate reader (Tecan).

mRNA extraction and quantitative Real-time PCR

Total RNA from indicated cell lines was extracted using Qiagen kit following the manufacturer instructions. The cDNA was amplified using takara's SYBR Green PCR master mix kit and the ABI 7900HT rapid real-time PCR system(Applied Biosystems) were used to measure mRNA expression levels of targeted genes. The GAPDH was utilized to normalize the quantification of gene expression.

Western blotting

Thermo Fisher Scientific's radioimmunoprecipitation assay (RIPA, 89900) buffer, which contains a protease inhibitor cocktail(*Roche*), was used to lyse the cells. Protein levels were measured using a Pierce bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific).10% SDS-PAGE and immunoblotting were used to electrophorese 20 μg of proteins. Proteins were transferred to membranes and probed overnight at 4°C with primary antibody. Then the membranes were washed in PBS buffer and incubated with secondary antibodies for 1 hour at room temperature. After washing, membranes were visualized through ECLUltra (New Cell and Molecular Biotech, China).

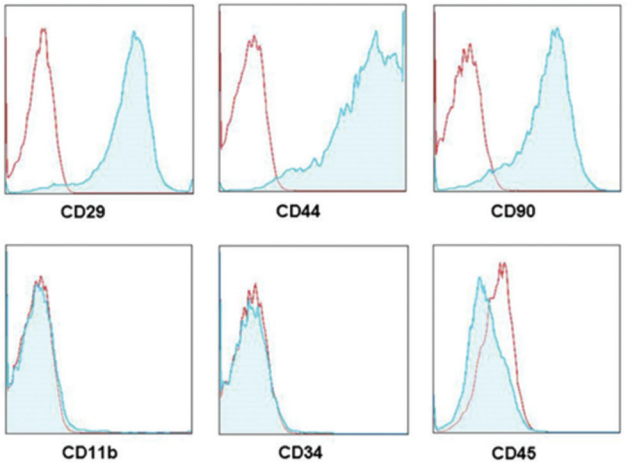
Transwell assay

According to the Boyden transwell assay was utilized to investigate the BM-MSCs' capacity for migration. 5×10^4 BM-MSCs were resuspended in 100 μL fresh medium with control serum (CS) or medicated serum (MS) injected into the top chambers and inoculated, and 5×10^4 HUVECs resuspended in 600 μL complete medium containing control serum or medicated serum added to the bottom chambers. After 12 hours of incubation at hypoxic conditions, The cells migrated to the bottom side of the membrane, washed with PBS and then they were fixed with 100% methanol, and washed with PBS, crystal violet solution (0.1% dilution). The upper side of the compartment was swabbed to eliminate the non-migrating cells, and a light microscope was used to count the migrating cells.

Statistical analysis

All findings are shown as mean ± SEM. Two-way analysis of variance (or Student's *t* test) with a Bonferroni, multiple comparison tests, was employed to examine the data when the means of more than two groups were being compared.

Figure 1. Characterization of the Human BM-MSCs Immunophenotype profile of human BM-MSCs. BM-MSCs were labeled with fluorophore-conjugated antibodies and analyzed by flow cytometry (blue histograms). BM-MSCs were positive for CD29, CD44, and CD90, and negative for CD11b, CD34, and CD45. BM-MSCs: bone marrow-derived mesenchymal stem cells.



GraphPad Prism software 7 (*GraphPad 7*) was used to analyze the data. $P < .5$ was considered statistically significant.

RESULTS

Isolation and identification of BM-MSCs

According to a prior report,[16] we selected the characteristics of human BM–MSCs. CD29, CD44, and CD90 were positively expressed but lacked myeloid cell marker CD11b, leukocyte marker CD45, and hematopoietic or endothelial progenitor cell marker CD34 (Figure 1A). The purity of the BM-MSCs was > 95%, which assured the numbers of MSCs from human bone marrow.

The medicated serum regulates the proliferation of BM-MSCs

To test the effects of RJQM on the proliferation of BM-MSCs, we used serum from rats pretreated with different concentrations of RJQM to stimulate BM-MSCs. After 48 h incubation, we observed that 10.8 $g \times kg^{-1} \times d^{-1}$ medicated serum dramatically increased the BM-MSCs growth compared with other concentrations (Figure 2A). The expression levels of genes associated with cell proliferation were then examined. It was observed that the expression of the genes encoding cyclin D1 and MYC were significantly increased when treated with RJQM medicated serum with peak expression elevation at 10.8 $g \times kg^{-1} \times d^{-1}$ RJQM concentration (Figure 2B). Since *cyclin D1* and *MYC* are the downstream target genes of the ERK1/2 signaling pathway[17,18], we conducted a western blot to track ERK1/2 phosphorylation and expression in comparison to the control group that treated with 0.9% NaCl solution. According to Western blotting results, protein expression was considerably higher, and the expression increased most significantly when the

Figure 2. RJQM Medicated promotes the growth of BM-MSCs through the ERK pathway. (A) Proliferation of BM-MSCs was measured using CCK8 assay (n = 5). The control group is a complete medium without medicated serum; (B) qPCR analysis of *CCND1* and *MYC* expression in BM-MSCs treated with medicated serum; (C) Expression of pERK1/2 and ERK1/2 in BM-MSCs was detected by Western blotting.

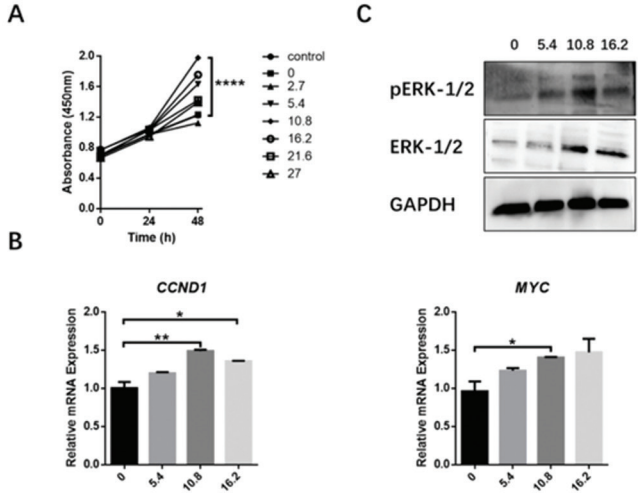
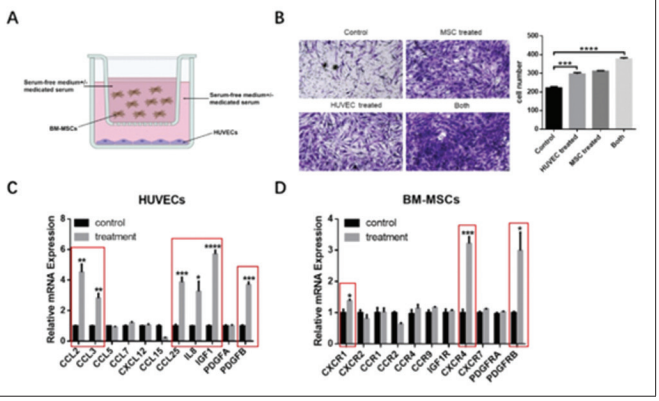


Figure 3. The medicated serum promotes the migration of BM-MSCs to HUVECs. (A) Diagram of the transwell co-culture system; (B) Transwell assay revealed the migratory cell number in BM-MSCs; (C) qPCR analysis of chemokine and cytokine expression in HUVECs; (D) qPCR analysis of receptor expression in BM-MSCs.



medicated serum was 10.8 g × kg^{-1} × d^{-1} (Figure 2C), which was consistent with the expression of *cyclin D1* and *MYC*.

The drug-containing serum promotes the migration of BM-MSCs

We performed transwell migration assays to further test the effects of medicated serum on the migration of BM-MSCs to endothelial cells. Treatment of either BM-MSCs or HUVECs alone or both for 48h three times with medicated serum aids in the spread of mesenchymal stem cells from the bone marrow to HUVECs (Figure 3A, B). Under hypoxic conditions, We then explored the effects of the medicine on the expression of chemokines/cytokines in HUVECs and the expression of their receptors in BM-MSCs, respectively. Hypoxic conditions are used to mimic diseased blood vessels of ASO.[19-21] qPCR results showed that the mRNA expression of CCL2, CCL3, CCL25, IL8, IGF1, and PDGFB increased dramatically after treatment with medicated serum (Figure 3C). CCL2, CCL3, and CCL25 have been reported to participate in the migration process of MSCs.[22] The gene expressions of the corresponding receptors for these up-regulated chemokines were detected in BM-MSCs, and it was found that CXCR1, CXCR4, CXCR7, and PDGFRB were up-regulated (Figure 3D). Since the SDF-1/CXCR4 axis has been well recognized for guiding mesenchymal stem cells (MSCs) to damaged areas,[23] the CXCR4 expression in BM-MSCs was also evaluated, However, the expression CXCL12 in HUVECs was not affected. qPCR data showed increased CXCR4 expression in BM-MSCs upon treatment of RJQM (Figure 3D). Together, these results indicate that medicated serum can stimulate bone marrow-derived mesenchymal stem cell migration to HUVECs by regulating chemokines surface receptors of BM-MSCs and increasing the secretion of chemokines by HUVECs.

DISCUSSION

RJQM is a clinically effective medicine to treat ASO,[24] but its mechanism was not fully understood. Our study suggests that the curative effect of RJQM on ASO may be achieved by promoting mesenchymal stem cell proliferation in the bone marrow and their migration to endothelial cells under hypoxia.

For summing up many years of clinical and scientific research experience, Professor Jiuyi Xi formulated RJQM for clinical use in treating ASO of the lower extremities showing ideal effects. Clinical observations have shown that RJQM can reduce blood lipids, improve vasoconstriction and relaxation, and protect vascular endothelium. It can eliminate the stress response in vasculitis and soften atherosclerotic plaques in blood vessels and promote patency of blood vessels[24]. Moreover, collateral circulation is established at the occlusion of arteriosclerosis to restore blood supply. The results of our study showed that RJQM-medicated serum can increase the growth of BM-MSCs through the ERK1/2 signaling pathway.

Moreover, drug-containing serum can push for the migration of BM-MSCs to HUVECs under hypoxic conditions. The qPCR results show that the receptor CXCR4, which is related to the homing function of MSCs,[25] is up-regulated. At the same time, the medicated serum also increased the chemokines CCL2, CCL3, CCL25, IL8, and IGF1 secreted by HUVECs. In particular, platelet-derived growth factor (PDGFB) in HUVECs and the corresponding receptor subunit PDGFRB in BM-MSCs were up-regulated, implying that this chemotactic pathway may play a critical role. Vascular endothelial cells that recruit BM-MSCs may aid in vascular healing and the development of collateral branches.

Here, our study indicates that the efficacy of RJQM treatment on ASO depends on the action of MSCs. RJQM treatment promoted the chemotaxis of MSCs and HUVEC via

the ERK pathway and might have a treatment effect on the ASO. In addition to ASO, MSCs are also involved in restoring other diseases. For example, MSCs can facilitate the repair of tubular epithelial by targeting multiple pathophysiological pathways, showing promising results for managing acute kidney injury.[26] Cardiac healing may be aided by the secretome of stem cells seen in human amniotic fluid and sustain endogenous regenerative mechanisms.[27] Notch-activated cardiac MSCs can promote myocyte proliferation and neovasculogenesis by secreting extracellular vesicles.[28] In dealing with knee osteoarthritis, paracrine signaling and recruitment of local endogenous cells allow MSCs to generate a repair microenvironment.[29] Clinical experiments employing MSCs have revealed that when transplanted into the articular environment, MSCs survive, proliferate, and begin healing via *neo*-cartilage production in *vivo*.[30-31] Further studies are needed to investigate the therapeutic effects of the RJQM formula on other diseases. Further studies are still needed to carry out the clinical experiment to verify the function of RJQM in the treatment of ASO.

Under hypoxic conditions, RJQM treatment can increase the secretion of chemokines in HUVECs, and an array of surface receptors are expressed on the BM-MSCs to facilitate the relocation of bone marrow mesenchymal stem cells to the ischemic site for repairing damaged blood vessels. Apart from regulating the chemotaxis of MSC, these chemokines also have other functions. For example, IL-8 stimulates MSC motility[32] and is a crucial protein in the inflammatory response, where it helps bring white blood cells and other immune cells to the scene of infection.[33] In addition, to promote MSCs to cross the vascular endothelium, CCL2, and CCL3 can recruit a wide variety of immune cells, including T cells, macrophages, B cells, and NK cells.[34,35] Furthermore, MSCs have been shown to stimulate the production of induced Tregs, which are as efficient by being naturally occurring Treg cells in their immunosuppressive roles.[36] Although Treg cells can inhibit T-cell activation, thereby reducing the development of atherosclerosis, they may also provide substantial protection against atherosclerosis by targeting APCs.[37] Therefore, the drug may affect the functions of other immune cells, thereby causing the body's immune response.

There are also limitations of this study. First, there were no experiments on animals, which can verify the opinions. Second, the results of this study might not be suitable for humans, further *in vivo* studies are needed to confirm our results.

CONCLUSION

RJQM derived from serum promotes the proliferation of BM-MSCs by up-regulating Cyclin D1 and MYC expression and the ERK pathway. Under hypoxic conditions, RJQM-medicated serum helps bone marrow-derived mesenchymal stem cells to migrate by up-regulating chemokine/cytokine receptors in BM-MSCs and chemokine/cytokine expression in HUVECs. This study only investigates the efficacy and mechanism of RJQM at the cellular level and needs to be verified *in vivo* in the future.

CONFLICT OF INTERESTS

No conflicts of interest exist, according to the authors, with the publishing of this work.

AUTHOR CONTRIBUTIONS

ZONG Y, ZHANG YK, XV YC, FANG YD, ZHAO C, and WANG YZ performed the research. ZONG Y, ZHANG YK, and XV YC analyzed the data. ZONG Y and ZHANG YK wrote the manuscript. CAO YM designed the research, reviewed the manuscript, and obtained funding. The essay was written collaboratively; each author read and agreed with the final draft. Zong Y and Zhang Y contributed equally to this work and share the first authorship.

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NON-STANDARD ABBREVIATIONS

ASO: arteriosclerosis obliterans; PADs: peripheral artery diseases; CAD: coronary artery disease; DM: diabetes mellitus; CHF: chronic heart failure; HUVECs: human umbilical vein endothelial cells; CLI: critical limb ischemia; CHF: chronic heart failure; ESRD: end-stage renal disease; RJQM: Ruan Jian Qing Mai formula; HSC: hematopoietic stem cell; MSCs: mesenchymal stem cells; BCA: bicinchoninic acid; CM: complete medium; CS: control serum; MS: medicated serum; *CCND1*: encoding cyclin D1.

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DATA AVAILABILITY STATEMENT

Without undue reluctance, the authors of this study will show the raw data used to support the conclusion presented here.

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