ORIGINAL ARTICLE

Secretome of hypoxia-preconditioned mesenchymal stem cells enhance the expression of HIF-1a and bFGF in a rotator cuff tear model

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ABSTRACT

Aim To determine the effect of secretome hypoxia mesenchymal stem cells (SH-MSCs) on the relative gene expression of hypoxia inducible factor-1a (HIF-1a) and basic fibroblast growth factor (bFGF) in accelerating histomorphometric repair of tendon to bone interface healing in rats acute rotator cuff tear (RCT) model.

Methods This is experimental research with posttest control group design. Thirty-male Wistar rats were divided into five treatment groups: healthy group and rotator cuff reconstruction group included four groups: SH-MSCs W2 (the treatment group was given a SH-MSCs 0.5 mL and terminated at weeks 2), NaCl W2 (the control vehicle group was assigned NaCl 0.5 mL and terminated at weeks 2), SH-MSCs W8 (the treatment group was given a SH-MSCs 0.5 mL and terminated at weeks 8), and NaCl W8 (the control vehicle group was given NaCl 0.5 mL and terminated at weeks 8). All the rats were terminated on day termination and HIF-1a and bFGF gene expression were analysed using qRT-PCR.

Results SH-MSCs significantly increased the HIF-1a and bFGF gene expression than the NaCl group even in week 2 and week 8. The highest increased gene expression of HIF-1a and bFGF was on week 8.

Conclusion SH-MSCs are important in the healing repair process of the tendon-to-bone interface in acute RCT model rats through increasing gene expression of HIF-1α and bFGF.

Key words: bFGF, HIF-1α, MSCs, rotator cuff tear

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INTRODUCTION

Rotator cuff tear (RCT) is a massive tendon injury mainly caused by traumatic injury and chronic tendon degeneration (1). Over 250,000 RCT repairs are performed per year, however repair failure affects >20% of patients (2). The failure rate increases up to 70 - 90% with chronic and large tears (3). A key reason for the high failure rate is the lack of healing at the repaired tendon-bone interface. In particular, robust repair requires functional reformation of the tendon-bone interface (4). In addition, the damaged tissue allows for a hypoxic niche in RCT characterised by the expression of hypoxia-inducible factor-1a (HIF-1a) thus prolonging the tendon reformation process (5). Recently, accelerated tendon reformation also requires various growth factors such as fibroblast growth factor-2 (FGF-2), transforming growth factor beta 1 (TGF-b1), and basic fibroblast growth factor (bFGF). Using growth factor to promote regeneration of the tendon, fibrocartilage, and bone at the tendon-bone interface is an emerging strategy in translational studies. The bFGF stimulates tenocytes resulting in increased collagen production and proliferation found in vitro. Previous studies reported that secretome from hypoxia-preconditioned mesenchymal stem cells (SH-MSCs) provide several growth factors that can promote tendon regeneration (8).

Mesenchymal stem cells (MSCs) have emerged as a promising candidate for essential tissue regeneration of rotator cuff tendon-bone healing (6). Bone marrow-derived MSCs (BM-MSCs) and adipose tissue-derived MSCs (AD-MSCs) improve collagen organization and collagen fibre coherence and enhance the tensile strength of tendons in a rat model of rotator cuff injury (7). A previous study reported that umbilical cord mesenchymal stem cells (UC-MSCs) can be used to recover tissue structure in a mouse model of ischemic injury and a C57BL6 mouse model of wound injury (8). However, using these MSCs entails invasive harvesting techniques, low collection efficiency, decreased ability with age, and short cell life span of MSCs. The secretome of MSCs can act as paracrine autocrine regulators to conduct intercellular information transmission and thereby regulate the immune response and tissue metabolism (9). Secretome secreted by MSCs play an important role in intercellular signal transduction and exosomes can regulate the tissue microenvironment and promote tissue repair and reconstruction (10,11).

Currently, there need for more research on the mechanism of exosomes in the process of tendon-bone healing. The diverse functions of secretome suggest that SH-MSCs may be involved in the regulation of tendon-bone healing. However, the effect of SH-MSCs on the Hif-1a and bFGF to promote tendon-bone healing after RCT is still unclear.

The aim of this study was to investigate effects of SH-MSCs on the relative gene expression of HIF-a and bFGF in accelerating histomorphometric repair of tendon-to-bone interface healing in rats acute RCT model.

MATERIALS AND METHODS

Materials and study design

This study was conducted in the Stem Cell and Cancer Laboratory Indonesia, from August 2022 until February 2023.

All the experimental procedures were performed with the approval of the Committee of Bioethics Universitas Padjajaran and Hasan Sadikin Hospital, Indonesia under the number 960/UN6.KEP/EC/2021 followed the Institutional Animal Care and Use Committee guidelines..

Animal experiment in vivo. Thirty male Wistar rats (8-week-old Wistar rats, male, 200-250 g) were used for in vivo experiment and divided into five groups, (1) healthy groups and rotator cuff reconstruction model included four groups: SH-MSCs W2 (the treatment group was given a SH-MSCs 0.5 mL and terminated at weeks 2), NaCl W2 (the control vehicle group was given NaCl 0.5 mL and terminated at weeks 2), SH-MSCs W8 (the treatment group was given a SH-MSCs 0.5 mL and terminated at week 8), and NaCl W8 (the control vehicle group was given NaCl 0.5 mL and terminated at week 8). Each group included 6 rats.

In the treatment group (SH-MSCs W2 and SH-MSCs W8), 0.5 mL of SH-MSCs was injected into the bony groove of the greater tuberosity in the rotator cuff repair area locally above the repair lesion during surgery before the skin was closed. While in the control vehicle group (NaCl W2

and W8), 0.5 mL of NaCl was given in the same procedure as treatment group. The surgical wound was then closed layer by layer according to the standard procedure of surgery with 4-0 nylon sutures. After the surgery, all animals were freed to move in the cage without immobilization and were checked daily for signs of general condition, including, normal activity, care, hydration status and nutritional status. Animals were housed in pairs and with free access to drinking water and food. The rats were sacrificed immediately 2 and 8 weeks after the surgery.

The rats were anesthetized with a lethal cocktail dose of Ketamine 50 mg/kgBW, Xylazine 10 mg/kgBW, and Acepromazine 2 mg/kgBW, which were injected intramuscularly and the supraspinatus and infraspinatus muscle-tendon units together with approximately 1.5 cm of the proximal humerus were sacrificed for molecular analysis.

Methods

MSCs isolation and culture. MSCs were derived from the female rats-umbilical cord at 19 days of pregnancy then were isolated and cultured as described previously (12–14). Briefly, the MSCs cells were cultured in the DMEM-low glucose medium (Gibco, USA) containing 10% foetal bovine serum (FBS, Gibco, USA), and 1% double antibiotics streptomycin and penicillin (Gibco, USA). The cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

MSCs characterization. The MSC surface markers were determined as described previously (15). Briefly, the cells at the 4th passage were detached and stained with anti-rat monoclonal antibodies including APC-conjugated CD73, FITC-conjugated CD90, PerCP-conjugated CD105, and PE-conjugated hemopoietic stem cell lineage Lin for 30 min at 4 °C. The labelled cells were analysed using flow cytometry (BD Accuri C6 PLUS; BD Biosciences, San Jose, CA, USA). The MSCs differentiation capacity was determined using osteogenic and adipogenic differentiation assay. Briefly, the cells were plated on 4×10^4 cells in 3.5 cm culture dishes under an osteogenic medium composed of DMEM High Glucose supplemented with 10% FBS, 1% Penstrep, 1 x 10^{-2} M sodium β-glycerophosphate, 1 x 10^{-4} M dexamethasone, and 5 x 10^{-5} M ascorbic acid. The medium was replaced every three days for 15 days. The calcium deposition showed a bright red colour after Alizarin Red staining Zigma (16–20). Adipogenic differentiation assay was analysed under Oil Red O staining assay. Briefly, adipogenesis was carried out in an adipogenic induction medium containing ADS medium supplemented with 0.5 mM isobutyl methylxanthine, 1μM dexamethasone, and 200 μMi ndomethacin. Experiments were performed at 15 and 28 days. Adipogenic differentiation was confirmed by oil-red-O staining.

HS-MSCs isolation. MSCs were ready to precondition using hypoxic chamber (Anaerobic Environment; ThermoForma, Waltham, MA, USA) when it reached 70% confluency in flask containing complete medium, then they were washed twice with PBS and incubated in DMEM for 24 h. MSCs were cultured in mix gas mixture composed of 5% CO2, 10% H2, and 85% N2 then maintained at 37°C. The oxygen level in the chamber was ~0.5%. After the incubation, SH-MSCs were collected by filtering the medium of MSCs using Tangential Flow Filtration (Formulatrix, USA) (21–23).

HIF-1a and bFGF gene expression analysis. Tissues of the supraspinatus and infraspinatus tendons attached to the major tubercle of the humerus bone's major tubercle were analyzed the quantitative real-time polymerase chain reaction (qRT-PCR) method. Total tissue RNA from tissues was extracted using TRIzol reagent (Invitrogen, Shanghai, China) according to the manufacturer's protocol. Briefly, first-stranded cDNA was synthesized with 1 ng of total RNA using Super-Script II (Invitrogen, Massachusetts, USA). SYBR No ROX Green I dye (SMOBIO Technology Inc, Hsinchu, Taiwan) was used for reverse-transcription in a real-time PCR instrument (PCR max Eco 48), and mRNA levels of the HIF-a (F:5'-GTCAACTGTGGAGCAACACG-3'; R:5'-CGTCAAAAGACAGCC ACTCA-3') and bFGF (F:5'- TGACAACTTTGGCATCGTGG-3': R:5'-GGGCCATCCA CAGTCTTCTG-3') were run using the respective primers. The thermocycler conditions used were as follows: initial step at 95 °C for 10 minutes, followed by 50 cycles at 95 °C for 15 seconds, and 60 °C for 1 minute. The gene expression was recorded as the cycle threshold (Ct). Data were obtained using Eco Software v5.0 (Illumina Inc, San Diego, CA, USA). All reactions were performed in triplicate, and data analysis used the $2^{-\Delta\Delta}$ Ct method (Livak method) (24).

Statistical analysis

All quantitative variables are expressed as mean and standard deviation (SD). The data obtained were collected, compiled and tested for normality with the Shapiro-Wilk test and homogeneity test with the Lavene test. Data analysis used one-way ANOVA and a least significant difference (LSD) comparison post hoc test $p<0.05$ indicated statistical significance.

RESULTS

MSCs characterization and differentiation

Our research revealed that MSCs have typical fibroblast-like, spindle-shaped cells with plastic adhering capabilities (Figure 1A). The differentiation capacity in the end of the fifth passage expansion was also examined. The standard and osteogenic media were given for 21 days during the MSC-like osteogenic differentiation experiment. Alizarin red dye staining was utilized to identify calcium deposits as a red appearance (Figure 1B). Moreover, the MSCs were successfully differentiated into adipogenic lineages while being stained with oil red-O (Figure 1C). Using flow cytometry, the immunophenotypical characteristics of MSCs were examined. A high level of CD90 (97.80±2.10%) and CD29 (96.70±0.66%) and low level of CD45 (2.00±0.75%) and CD31 (4.60±2.34%) was found (Figure 1D).

SH-MSCs induced HIF-1a gene expression on acute RCT model

To determine the effects of SH-MSCs Group and NaCl vehicle Control Group on HIF-1α gene expression in acute RCT model rats, we observed at 2 different times, i.e., the two, 2nd and 8th week after the surgery and therapy administration. SH-MSCs significant increase the of HIF-1a gene expression up to 4.23±1.75-fold change compare to NaCl group for 2 weeks was found. Interestingly, in 8-week SH-MSCs dramatically induced HIF-1a gene expression up to 16.45±4.39-fold change compare to NaCl group (Figure 2).

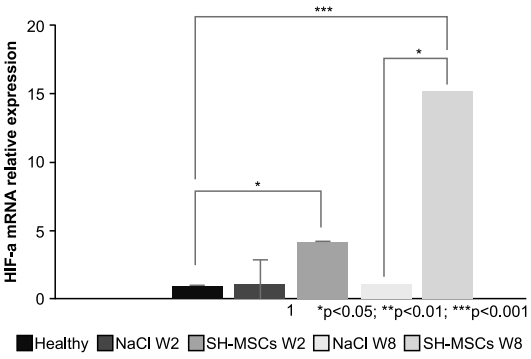

Figure 2. The effect of secretome hypoxia mesenchymal stem cells (SH-MSCs) on HIF-1a gene expression on acute rotator cuff tear (RCT) model. RNA was extracted from tendon-bone of each group and analysed for mRNA expression by qRT-PCR (n=6±SD). Data are presented as fold change in gene expression relative to healthy group
*statistically significant

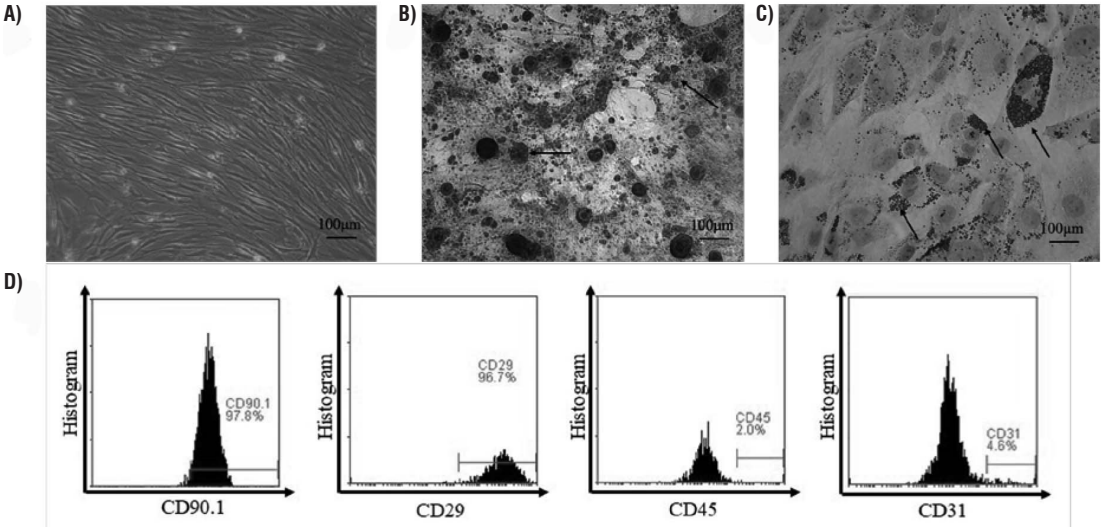

Figure 1. A) MSCs characterization and differentiation. The black arrow presented the fibroblast-like cells (magnification10x, scale bar 100 μm); B) MSC differentiation. A red bright colour marked by the black arrow in a response to the calcium deposition in osteocyte-differentiated MSCs via staining by Alizarin red (magnification 40x, scale bar 50 μm); C) The red colour marked by the black arrow in a response to the lipid deposition under Oil-red O staining; D) The surface marker analysis of the expression of CD90, CD29, CD45, and CD31

SH-MSCs induced bFGF gene expression on acute RCT model

SH-MSCs significantly increased the bFGF gene expression up to 5.77±1.47-fold change compared to NaCl group for 2 weeks. Interestingly, in 8-week SH-MSCs dramatically induced bFGF gene expression up to 9.86±2.07-fold change compared to NaCl group (Figure 3).

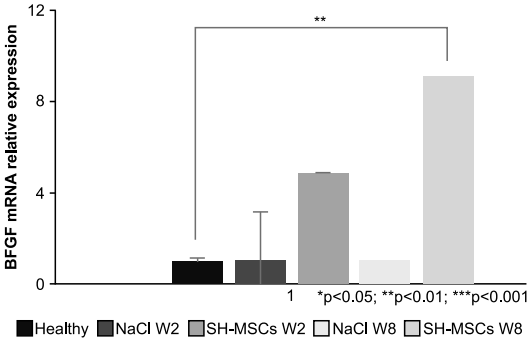

Figure 3. The effect of secretome hypoxia mesenchymal stem cells (SH-MSCs) on bFGF gene expression on acute rotator cuff tear (RCT) model. RNA was extracted from tendon-bone of each group and analysed for mRNA expression by qRT-PCR (n=6±SD). Data are presented as fold change in gene expression relative to healthy group
*statistically significant

DISCUSSION

The ability of MSCs to perform immunomodulation and angiogenesis makes them strong candidates for tissue repair therapy, including tendon-bone repair (25–28). During the healing process of bone defects, a hematoma is first formed around the wound; growth factors are activated, and stem cell recruitment, proliferation and differentiation are activated (29). Bone repair occurs under hypoxic conditions, HIF-1α is indispensable in bone repair. Several studies reported that MSCs under hypoxic conditions can increase HIF-1α expression (30,31). This phenomenon indicates that under hypoxic conditions, MSCs have better survival ability due to increased transplantation effectiveness and immunomodulation function (32,33). A previous study also reported that the hyper baric oxygen therapy or platelet rich plasma can improve the tendon repair or bone repair (27). In this study, it was found that SH-MSCs administration significantly increased HIF-1α expression and was correlated with bone repair. The results of our study are in line with previous studies that there was a significant increase in HIF-1α gene expression and a decrease in cell apoptosis after administering exosomes MSCs in rats with bone fracture models (32,34). A previous study also reported that knockdown of HIF-1α *in vivo* and *in vitro* significantly impair bone regeneration and osteogenesis of periosteum-derived mesenchymal stem cells, indicating the indispensability of the HIF-1α in bone regeneration under hypoxia (5,35). Because HIF-1α is degraded by proline hydroxylase under normoxia. Several cytokines and growth factors in SH-MSCs can activate osteogenesis of osteoblast-precursors through integrin receptors and Wnt-β-catenin signalling pathway (36). Hif-1α has also been reported to bind β-catenin and form the hif-1α/β-catenin complex, accompanied by increased hif-1α transcriptional activity leading to cell survival and proliferation (37).

This study also showed that the administration of SH-MSCs can increase the expression of the bFGF gene which has an impact on improving the histomorphometric healing of the tendon to bone interface in the rat model of acute RCT. Previous study reported that increased bFGF gene expression leading to improved Achilles tendon healing in rabbit (38). Administration of the bFGF gene to the injured Achilles tendon will increase tendon strength through increased collagen, biomechanical resistance and increased angiogenesis which plays a role in wound healing (39). In addition, the mechanism of accelerated wound healing is increased by the bFGF gene due to a shorter regeneration process, stronger tissue and better histology (40). The increased level of bFGF gene to the injured Achilles tendon will increase tendon strength through increased collagen level, biomechanical resistance and increased angiogenesis, which plays a role in wound healing (41). SH-MSCs contained several bioactive factors such as EGF, HGF and bFGF that have protective properties in wound healing could activate the PI3K/Akt and/or FAK/ERK1/2 signalling pathway (42–44). The activation of PI3K pathway was ameliorating the cell migration or proliferation through epithelial mesenchymal transition (EMT) form leading to wound healing (45,46).

In conclusion, SH-MSCs are important in the healing repair process of tendon-to-bone interface in acute RCT model rats through increasing gene expression of HIF-1α and bFGF. Those genes play indispensable roles in osteogenesis under hypoxic

microenvironment. These findings suggest that SH-MSC is a promising therapy for tendon-to-bone tissue engineering, and HIF-1α serves as a potential target to promote bone repair, which lays a solid foundation for tendon-to-bone regeneration.

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TRANSPARENCY DECLARATION

Conflict of interest: None to declare

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