Topical Gel Containing TNF-α-Activated Mesenchymal Stem Cell Secretome (MSC-CM-T) Increases *HMGB1* Expression and Promotes Wound Healing in Full-Thickness Skin Defect Animal Model

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ABSTRACT
Mesenchymal stem cell conditioned medium or secretome contained several growth factors and cytokines that promote wound healing, thus topical gel containing TNF-α-activated MSC secretome (MSC-CM-T) may be considered as a promising candidate for the treatment of cutaneous wounds. This study aimed to investigate the benefit of MSC-CM-T in promoting wound healing by evaluating changes in wound closure percentage and *HMGB1* gene expression. Eighteen Wistar rats were randomly assigned to treatment group receiving 200 µL MSC-CM-T topical gel (9 subjects) and control group receiving 200 µL serum-free DMEM topical gel (9 subjects). A 6 cm incision wounds were made on their backs, and treatment were given twice daily for 6 days. Wound closure were measured using standard calliper, and *HMGB1* gene expression were evaluated using quantitative Real-Time PCR. The treatment group showed higher increase of wound closure percentages (Δ wound closure 46,11±4,41 *vs* 20.57±6.87 %; $p<0.001$), *HMGB1* expression on day 3 (11.63±0.28 *vs* 10.83±0.31 fc; $p<0.001$) and day 6 (12.73±0.29 *vs* 11.87±0.31 fc; $p<0.001$), and Δ *HMGB1* expression between day 0–3 (2.65±0.66 *vs* 1.81±0.69 fc; $p<0.05$) and day 0–6 (3.74±0.77 *vs* 2.85±0.72 fc; $p<0.05$). This study also found a strong positive correlation between wound closure percentages and *HMGB1* expression on both day 3 ($r = 0.7231$; $p<0.001$) and day 6 ($r = 0.7262$; $p<0.001$). Based on these results, it can be concluded that MSC-CM-T may potentially promote faster wound healing, with HMGB1 as one of the possible factor that mediates this effect.

Introduction

The integrity and function of injured skin can be restored to normal conditions through the wound healing process.[1] Various complications due to abnormalities in any wound healing phases or longer wound healing period may increase morbidity and mortality, hence wounds should be adequately healed within a relatively short time.[2] Wound closure rate is the main parameter to be considered when assessing the quality of the wound healing process and the risk of scarring or complications such as infection.[3] Currently, there are many novel treatment options, such as stem cell therapy, that can help to promote the wound healing process while also preventing unwanted complications.[3–5]

Mesenchymal stem cells (MSCs) have been reported to accelerate the acute wound healing process by inducing a regenerative healing process while reducing the formation of excessive scar tissue.[3,6] MSC secretome or conditioned medium (MSC-CM) contains many different soluble factors that can play a role in the wound healing process, including growth factors (PDGF, PGE2, TGF-β, IGF-1, G-CSF, GM-CSE, VEGF and KGF), inflammatory proteins (TNF-α, IL-1, IL-8, IL-10, IL-6, LIF, IL-11, MCP-1, PGE2, IL-9 and IL-13), ECM protein (TIMP-1, TIMP-2, MMP-1, MMP-2, MMP-3, MMP-7, ICAM, collagen, elastin, decorin, and laminin), and angiogenic factors (PDGF, VEGF, MCP-1, TGF-β1, FGF, EGE, CXCL5, TGF-α, Ang-1, and Ang-2). The bioactive compounds within the MSC-CM are known to accelerate the re-epithelialization process, reduce inflammation, induce angiogenesis, and increase collagen production.[3,5]

Mesenchymal stem cell conditioned medium (MSC-CM) can be optimally conditioned through culture media under TNF-α stimulation to increase the amount of paracrine factors produced and trigger the release of additional types of paracrine factors that can help increase the regenerative potential of MSC-CM.[4] Secretome obtained from cell culture with TNF-α stimulation were found to induce faster wound closure compared to secretome without preconditioning. Secretome with TNF-α preconditioning also showed significantly higher levels of FGFb, PDGF-BB, IL-6, VEGF and MMP-13, thus helping to provide a microenvironment that is more supportive of the wound healing process, increase angiogenesis, keratinocyte migration and reepithelialization.[7]

High mobility group box 1 (HMGB1) is a type of damage associated molecular pattern (DAMP) protein that plays an important role in the wound healing process,[8] and also being considered as one of the possible facilitating factors that are related to the secretome-mediated wound healing.[9] HMGB1 interacts with a number of different receptors to regulate cellular responses related to tissue repair and regeneration processes, including immune system activation, cell migration, cell growth, stem cell recruitment to the wound area, and stem cell proliferation.[10]

This study aims to evaluate the effect of topical MSC-CM-T gel treatment on the healing process of a full-thickness wound defect in a Wistar rat model by assessing changes in the percentage of wound healing during treatment period and *HMGB1* gene expression.

Methods

Full-thickness Wound Defect Models

Eighteen white Wistar rats (*Rattus norvegicus*) between the age of 10-12 weeks were randomly divided into one of 2 groups, the control group (9 rats) or treatment group (9 rats). All subjects were kept in the same conditions during the study period, with a constant room temperature of 28 °C, 40-60% humidity, and a 12 hour light-dark cycle. There were no significant difference in average initial body weight or change in body weight between the two research groups during the study period. No complications such as injury, illness or death were reported during the study period.

The rats were anesthetized by IM ketamine injections (7 mg/kg body weight), then the rats were placed on a surgical board in a prone position and all four legs were tied. The hair on the back of the rats was shaved clean, followed by an aseptic procedure using povidone iodine solution. Then a 6 cm full-thickness incision was made in each rat.

MSC-CM-T Gel Preparation

MSC was cultured with a cell density of 1.5×10^4 cells/well. Cells were cultured in a 24-well plate with standard media containing DMEM, supplemented with 10% FBS, 1% penicillin (100 U/mL)/streptomycin (100 µg/mL), and 0.25% amphotericin B at 37°C and 5% CO_2 levels. After achieving 95% confluence, the standard media was then aspirated and replaced using osteogenic differentiation media containing Mouse MesenCult™ Osteogenic Differentiation Basal Medium with Mouse MesenCult™ Osteogenic Differentiation 5X Supplement 20%, 1% L-Glutamine, 1% penicillin (100 U/mL)/streptomycin (100 µg/mL) and 0.25% amphotericin B. This differentiation media was renewed every 3 days.

The MSC culture in serum-free DMEM media (1×10^4 cells/well) were then supplemented with 10 ng/ml recombinant TNF-α, and then incubated at 37°C and 5% CO_2. After 24 hours, MSC-CM was separated using a centrifugation process at 1900 rpm for 10 minutes. Topical MSC-CM-T gel for the treatment group was made using the base gel combined with 200 µL of MSC-CM-T, and for the control group, the base gel was combined with 200 µL of serum-free DMEM. The topical gel treatment was given twice daily for 6 days.

Wound Defect Healing Assessment

Wound length was measured daily using a standard caliper. Wound closure percentage was evaluated on day 3 and 6 using the formula (A0–At)/A0 × 100%, where A0 is the initial wound length, while At is the wound length on day 3 (A3) and 6 (A6).

HMGB1 Gene Expression Quantification

The *HMGB1* gene expression in every study subjects was evaluated on day 0, 3 and 6. The venous blood for RNA extraction was obtained from tail vein sampling. After extraction, the concentration and purity of total RNA was determined using NanoDrop2000 spectrophotometer (Thermo Fisher Scientific, USA). Purity ranging between 1.8 and 2.1 is considered as good RNA quality. The mRNA expression of the *HMGB1 gene* was determined by Realtime PCR. After RT-PCR examination, gene amplification was quantified by determining the cycle threshold (Ct).[11,12]

Statistical Analysis

Statistical analyses were performed using SPSS software version 23.0 (IBM Corp., USA). Values were expressed as mean±SD. Comparison between groups were analyzed using unpaired t-test, and correlation between study variables were analyzed using Spearman's correlation test, with *p* value of <0.05 as the significance threshold.

Results

Wound Closure Percentage

Mice in the treatment group that received MSC-CM-T gel therapy showed significantly better wound closure rate compared to the control group that received serum-free DMEM gel, with greater increase of wound closure percentage between day 1 – 6 in the MSC-CM-T gel group compared to the control group (46.11 ± 4.41 *vs* 20.57 ± 6.87 %; *p* <0.001). Changes in the wound closure percentage from the beginning to the end of the study period can be seen in Table 1 and Figure 1.

Table 1. Wound closure percentage in control and treatment groups*

Variables	Control (n=9) (Serum-free DMEM)				Treatment (n=9) (MSC-CM-T)				*p*
	Mean	SD	Min	Max	Mean	SD	Min	Max	
Day 1	3.32	3	0	10	3.15	2.42	0	6.7	0.864
Day 2	5.35	2.87	3.3	11.7	5.56	2.21	1.7	8.3	**0.025**
Day 3	7.60	3.34	3.3	13.3	10.92	2.23	8.3	15	**0.002**
Day 4	10.74	4.57	3.3	18.3	18.52	4.43	11.7	26.7	**<0.001**
Day 5	17.03	5.88	8.3	25	33.88	8.08	23.3	48.3	**<0.001**
Day 6	23.90	7.60	15	31.7	49.26	12.84	33.3	71.7	0.668
Δ Day 1–6	20.57	6.87	11.7	30	46.11	4.41	26.6	66.7	**<0.001**

*Unpaired t-test, significant when *p* <0.05; values presented as percentage (%).

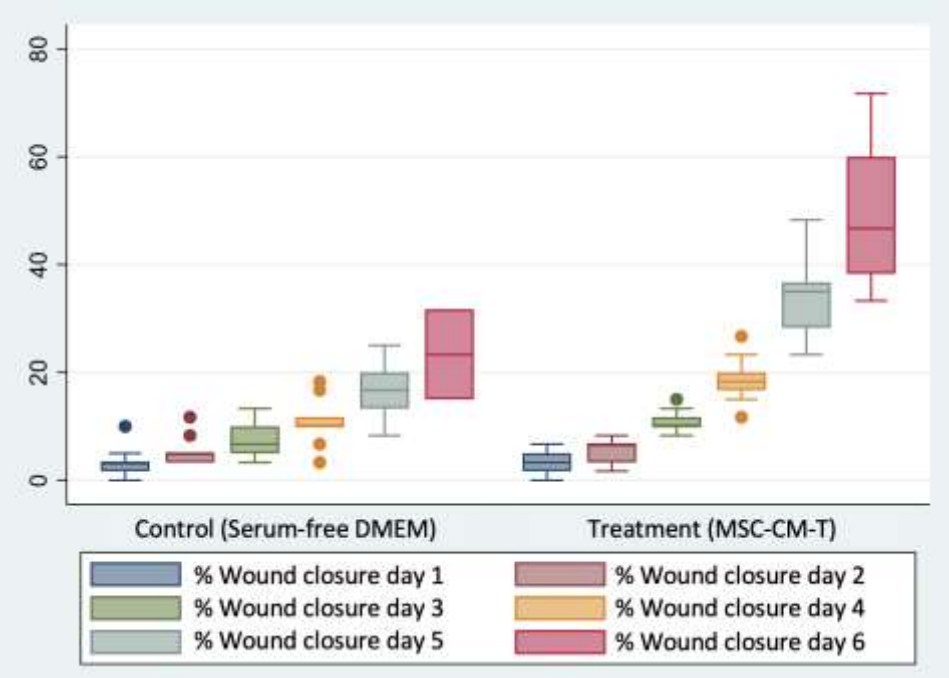

Figure 1. Comparison of wound closure percentage between control and treatment group.

HMGB1 Expression

There was no significant difference in basal *HMGB1* expression between the treatment and control groups (mean expression of 8.98 ± 0.73 and 9.02 ± 0.68-fold change, respectively; $p = 0.918$). During the study period, the MSC-CM-T group showed significantly higher *HMGB1* expression than the control group, both on day 3 (11.63 ± 0.28 *vs* 10.83 ± 0.31-fold change; $p < 0.001$) and day 6 (12.73 ± 0.29 *vs* 11.87 ± 0.31-fold change; $p < 0.001$). The MSC-CM-T group also showed significantly higher mean increase in *HMGB1* expression compared to the control group, both between treatment day 0–3 (2.65 ± 0.66 *vs* 1.81 ± 0.69-fold change; $p < 0.05$) and between treatment day 0–6 (3.74 ± 0.77 *vs* 2.85 ± 0.72-fold change; $p < 0.05$). The analysis also found a strong positive correlation between wound closure percentage and *HMGB1* expression both on treatment day 3 ($r = 0.7231$; $p < 0.001$) and day 6 ($r = 0.7262$; $p < 0.001$). Comparison of *HMGB1* expression between treatment and control group from the beginning to the end of the study period can be seen in Table 2 and Figure 2.

Table 2. Expression of *HMGB1* mRNA in control and treatment groups*

Variables	Control (n=9) (Serum-free DMEM)				Treatment (n=9) (MSC-CM-T)				*P*
	Mean	**SD**	**Min**	**Max**	**Mean**	**SD**	**Min**	**Max**	
Day 0	9.02	0.68	8.16	9.94	8.98	0.73	8.08	10.12	0.918
Day 3	10.83	0.31	10.2	11.2	11.63	0.28	11.2	12.11	**<0.001**
Day 6	11.87	0.31	11.3	12.2	12.73	0.29	12.2	13.12	**<0.001**
Δ Day 0–3	1.81	0.69	1.06	2.99	2.65	0.66	1.81	3.6	**0.019**
Δ Day 0–6	2.85	0.72	1.51	3.76	3.74	0.77	2.36	4.59	**0.022**

*Unpaired t-test, significant when $p < 0.05$; values presented as fold change.

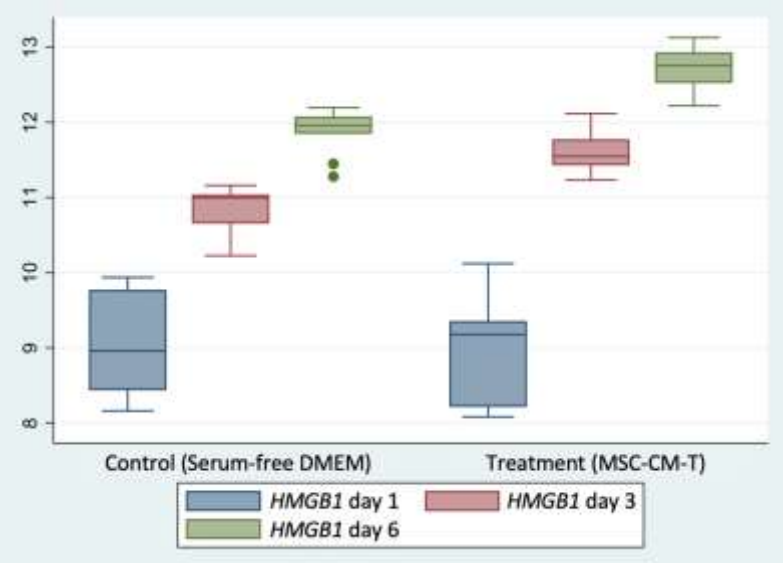

Figure 2. Comparison of HMGB1 expression between control and treatment group.

Discussion

Ideal wound care are expected to accelerate the healing process of acute wounds and prevent various complications such as secondary infections, chronic wounds, or the formation of scar tissue.[3] Mesenchymal stem cell conditioned medium contains various types of bioactive compounds that are known to support the tissue repair and regeneration process,[13,14] thus can be considered as one of the alternative treatment options to improve the wound healing process,[3–5] specifically by accelerating the healing process of acute wounds and inducing a regenerative healing process while reducing the formation of excessive scar tissue.[3,6]

This study found that topical MSC-CM-T gel can increase full-thickness wound closure rate in animal models, which was indicated by a significantly greater increase in the percentage of wound closure compared to controls that were given topical serum-free DMEM gel. This is in line with the results published by a number of previous studies that also reported significant increase in wound closure rate after MSC-CM treatment, such as studies from Xiao *et al.*[15] and An *et al.*[16] that evaluated MSC-CM from human adipose tissue and Zhu *et al.*[17] and Fang *et al.*[18] that evaluated MSC-CM from human umbilical cord tissue. Other studies using MSC-CM from rat adipose tissue also found similar results, such as Shiekh *et al.* that reported an increase in wound closure rate on day 4, 8, and 14, accompanied by increased re-epithelialization, epithelial maturation, granulation tissue formation, type I collagen deposition, and angiogenesis on day 14,[19] and Putra *et al.* that reported an increase in wound closure rate on day 6 accompanied by a significant increase in PDGF levels on days 3 and 6.[20] Overall, the results of this study and several previous studies on the topic have highlighted the possible benefit from topical MSC secretome treatment to accelerate the wound healing process.

One mechanism that is thought to play a role in facilitating the effects of the secretome in accelerating the wound healing process is the release of HMGB1.[21] This study found a trend of increased *HMGB1* expression in both treatment and control groups during the study period, thus emphasizing the potential role of this *damage-associated molecular pattern* (DAMP) protein in the wound healing

process.[9] HMGB1 interacts with various receptors to regulate cellular responses involved in tissue repair and regeneration process, ranging from activating the immune system, increasing viability, proliferation and migration of fibroblasts and keratinocytes,[8] to recruiting stem cells to the wound area and inducing their proliferation.[10]

This study found a positive correlation between wound closure percentage and *HMGB1* expression on day 3 and 6, which means that the wound closure percentage will increase along with the increased expression of *HMGB1*. The MSC-CM-T group also showed greater increase in *HMGB1* expression compared to the serum-free DMEM group, thus supporting possible role of HMGB1 as one of the factors mediating the effect of MSC-CM-T in accelerating wound healing. Evidence supporting this notion was also reported in previous studies. The administration of MSC-CM from bone marrow cultured in hypoxic conditions was found to induce HMGB1 production,[9] dan HMGB1 that were released in the skin graft wound healing area were found to mobilize the cell population with PDGF receptors (PDGFRα^+) to regenerate injured epithelium.[22]

HMGB1 in the wound area will bind to receptors such as toll-like receptors (TLR 2, 4 and 9) and receptors for advanced glycation end products (RAGE) to activate the local inflammatory response.[8–10] This pro-inflammatory effect is mediated by the *mitogen-activated protein kinases* (MAPK) and *nuclear factor kappa B* (NF-κB) pathways,[8,23] and will eventually increase the expression of a number of cytokines such as VEGF, IL-1α, IL-1β, IL-4, IL-6, IL-8, IL-9, IL-10, IFN-γ, GM-CSF dan TNF-α from monocytes, macrophages and neutrophils in the wound area.[23,24] HMGB1 also acts as a chemoattractant factor that mediates the migration of monocytes and neutrophils to the wound area.[23,25] In the proliferation phase, HMGB1 are known to promote re-epithelialization, granulation tissue formation, fibroblast proliferation, and act as a profibrogenic molecule to induce collagen formation, reduce MMP-1 expression, and increase TIMP-1 expression, thus further promoting the wound healing process.[8,25]

Conclusion

The results of this study emphasize the potential role of MSC-CM-T in promoting faster wound healing, with HMGB1 as one of the factors mediating this effect. However, further studies are required to evaluate the benefits of topical MSC-CM-T gel treatment for wound healing in humans, while evaluating the feasibility and cost-effectiveness of using this novel treatment option routinely in daily clinical practice.

Acknowledgments

Conflict of interest

The authors report no conflict of interest.

Ethical approval

This study has been approved by The Ethics Committee for Research at the Hasanuddin University Medical Faculty in Makassar, Indonesia.

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