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Secretome MSCs restore α-Smooth Muscle Actin Protein Tissue Expression in Croton Oil-Induced Hemorrhoid rats

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ABSTRACT

Background: Hemorrhoidal disease, a prevalent and distressing condition affecting a significant number of the population, presents a considerable challenge in both clinical management and patient quality of life. Secretome mesenchymal stem cell hypoxia (S-HMSCs) is involved in accelerated remodeling and regeneration of wound tissue, including hemorrhoids, through anti-inflammatory and anti-fibrotic molecules paracrine activities. **Objective:** This study aims to investigate the effect of Secretome Hypoxia MSCs (S-HMSCs) in restoring α-smooth muscle actin (α-SMA) expression in croton oil-induced hemorrhoid rats. **Material and Methods:** An experimental study with a post-test-only control group design was used in this study. Croton oil was administrated for inducting hemorrhoidal disease. A total of 24 male Wistar rats were divided into four groups (n=6); Sham (Healthy group); Untreated (Croton oil+NaCl 300 µL) Croton oil+S-HMSCs 150); Secretome 150 µL (Croton oil+S-HM SCs 150 µL) and Secretome 300 µL (Croton oil+S-HMSCs 300 µL). S-HMSCs were injected intraperitoneally every week for up to 4 weeks. All animals were scarified and the rectal tissue was collected for α-SMA immunohistochemical staining analysis. **Results:** After hemorrhoid induction, α-SMA was expressed 20% higher than Sham group, furthermore, administration of 150 µL and 300 µL of S-HMSCs may decreased by 15% and 20% α-SMA expression compared to the Untreated group, expression in croton oil-induced hemorrhoid rats.

Keywords : Secretome, TGF-β, IL-10, Myofibroblast, Hemorrhoid.

INTRODUCTION

Hemorrhoids are one of the oldest anorectal ailments that are commonly identified, and extensively researched, and have even become one of the world's most popular health issues[1,2]. Hemorrhoids are estimated to afflict 10 million people in the United States, and with a prevalence of 4.4%, they are the third most frequent diagnosis at urgent care and outpatient patient visits[3,4]. In Indonesia, the prevalence of hemorrhoids is estimated to be 5.7%, affecting 12.5 million people. However, only 1.5% of cases are diagnosed, as hemorrhoid sufferers frequently feel ashamed to seek care and choose to conceal their condition instead[5]. There are numerous different ways to treat hemorrhoids, but up until recently, the data supporting many of these methods was inadequate. Some of these therapy methods have withstood the clinical test of time despite the lack of scientific proof[4,5]. Therefore, hemorrhoidal disease treatment presents a considerable challenge in both clinical management.

Recent advancements in regenerative medicine have spotlighted the potential of stem cell-based therapies in mitigating tissue dysfunction and promoting healing. Specifically, stem cell therapies involving human mesenchymal stem cells (MSCs) have demonstrated immunomodulatory and regenerative capabilities, implicating their potential as therapeutic agents for conditions involving tissue remodeling and inflammation. In controlled hypoxia condition, MSCs exhibit higher rates of proliferation and better retain their stemness properties trough higher secreting robust potent soluble molecules compared with normoxia[6,7]. The collective term for the soluble substances produced by stem cells in hypoxic conditions is secretome mesenchymal stem cell hypoxia (S-HMSCs)[8–10]. In addition to cellular proteins, growth factors, angiogenic factors, hormones, extracellular matrix proteins, and even some lipid mediators and genetic material, their secretions comprise a variety of cytokines and chemokines[11–13].

Hemorrhoid healing is characterized by a number of dynamic processes and complicated changes in the rectal tissue milieu, such as the control of inflammation, vasodilatation, and tissue regeneration[3,14,15]. In regard to regulating inflammation, the main anti-inflammatory cytokines, IL-10 and TGF-β, synergistically regulate inflammation microenvironment of hemorrhoid tissue leading to controlled vasodilatation[16–18]. The last stages of hemorrhoid healings, tissue remodeling and regeneration, are the crucial phase that coordinate the process of ECM remodeling and cell proliferation and differentiation[19,20]. Among the key players in tissue remodeling and contractile functions is the alpha-smooth muscle actin (α-SMA) protein, which is indicative of myofibroblast activation-a process implicated in wound healing and fibrosis[17,21]. Understanding the dynamics of α-SMA expression in the context of hemorrhoid induction and its potential modulation by therapeutic interventions is crucial for advancing our management strategies for this condition.

Besides anti-inflammatory, IL-10 and TGF-β also have anti-fibrotic effects that intersect with the myofibroblast activation pathway[22,23]. Recent studies have unveiled their additional role in controlling myofibroblast activity and α-SMA expression, presenting a novel approach to counter fibrosis[24,25]. IL-10, known for its immunosuppressive effects, has been shown to directly inhibit myofibroblast differentiation by attenuating TGF-β signaling and by antagonizing TGF-β-induced SMAD signaling, modulates the intensity and duration of TGF-β signaling, preventing exaggerated myofibroblast activation which is consequence with α-SMA expression[26,27]. Building upon this foundation, our investigation explores the effects of S-HMSCs (stem cell-derived human mesenchymal stem cells) on α-SMA expression within hemorrhoidal tissue. By quantifying changes in α-SMA expression levels following S-HMSC administration, we aim to unravel the potential of stem cell-based interventions in restoring tissue homeostasis and ameliorating the aberrant α-SMA expression observed in hemorrhoidal disease.

METHODS

Secretome Isolation and Differentiation Assays

Under aseptic conditions, the MSCs were extracted from the umbilical cord of a healthy Wistar rat between days 19 and 21 of gestation and cultivated in a plastic flask[28]. The tissue explants were grown at 37°C and 5% oxygen within an expansion medium (EM) containing 10% fetal bovine serum/FBS (Gibco, 10270106, South America), 100 IU/mL penicillin-streptomycin (Sigma-Aldrich), and Dulbecco's Modified Eagles Medium/DMEM (Gibco, 11885084, NY, USA). The EM is switched out twice weekly until the cell reaches 80% confluence.

After being split and stained for 30 min at 4°C with anti-rat monoclonal antibodies, APC-conjugated CD73, FITC-conjugated CD90, PerCP-conjugated CD105, and PE-conjugated hemopoietic stem cell lineage, the cells in the fourth passage went through analysis using BD Accuri C6 PLUS flow cytometry (BD Biosciences, San Jose, CA, USA). Additionally, to generate Hypoxia-

MSCs, the MSCs were maintained for 24 hours at 37°C and 5% O2 in a hypoxic chamber (STEMCELL Technologies, Biopolis, Singapore). The collected media was gathered and filtrated via the TFF procedure with a molecular weight threshold of 10–50 kDa in order to produce S-HMSCs.

Hemorrhoid Rat Models

All research protocols were carried out in compliance with bioethics standards put forth by the SCCR Laboratory. The experimental protocol was approved by the Bioethics Commission of the Faculty of Medicine, Universitas Sebelas Maret, Indonesia. Wistar rats, used as laboratory animals, were obtained from Laboratorium Penelitian dan Pengujian Terpadu (LPPT), Universitas Gadjah Mada and acclimated for five days. The animals were given unlimited access to food and water. The animals were kept in conditions with a 12-hour light/dark cycle, consistent humidity, and temperature that were measured with thermohydrometer. Temperature and humidity readings ranged from 20 to 22 C and 35% to 45%, respectively. Following experimentation, the animals were put to sleep in accordance with accepted practices by receiving a 200 mg/kgBW overdose of thiopental sodium intraperitoneally.

Except for the normal control group, all the groups were given a croton oil mixture (deionized water, pyridine, diethyl ether, and 6% croton oil in diethyl ether in the ratio of 1: 4: 5: 10), which was used to induce hemorrhoids. After all of the animals had fasted for the previous night, sterile cotton swabs (4 mm in diameter) were placed into their anus (the part of the anus that is located 20 mm from the anal entrance) and held there for 10 seconds. Up to 7 to 8 hours after using the croton oil, a linear progression of the edema was seen. By calculating the amount of Evans Blue (EB) dye, the croton oil-induced plasma exudation in the rat recto-anal section was evaluated quantitatively. The animals received an injection of EB dye (30 mg/kg) into their tail veins 30 minutes before the croton oil preparation was applied to generate hemorrhoids.

a-SMA Immunohistochemistry

For a-SMA IHC staining, the slide was carried out for deparaffinization by thoroughly submerging it in xylene and followed by rehydration through absolute ethanol, ethanol 90%, ethanol 80%, and ethanol 70%. The next step is antigen retrieval, which involves soaking the slide in citrate buffer 1x pH 6.5 and putting it in the Decloaking Chamber (BIOGEAR). The slides were then treated with two blocking procedures: endogenous peroxidase blocking with H2O2 and background blocking by coating Background Sniper (Star Trek Universal-HRP Detection Kit) solution on the slide. The first antibody α-SMA (1:100, Abcam, Cambridge, UK) was then incubated overnight at 4°C. It was then counterstained by dipping it in Mayer Hematoxylin (Bio-Optica Milano S.p.A.) before the Trekkie Universal Link secondary antibody was administered (Starr Trek Universal-HRP Detection Kit, Cat. No. STUHRP700L10- KIT, Biocare Medical). For the last, the clearing process was carried out by fully dipping the slide in xylene and then mounting. The α-SMA expressions were counted under microscope and the percentage was calculated using imageJ.

Statistical Analysis

Each data was studied with SPSS version 22.0 (IBM, Armonk, NY, USA) and are shown as means standard deviation (SD). To assess group differences, Student's t-tests, one-way ANOVA. Statistical significance was set at p 0.05.

RESULT

MSC Characterization

The morphological characteristic of isolated cells under microscopical observation was demonstrated by their plastic adherent, homogenous shape, typical MSC fibroblast-like phenotype,

and round nucleus. We proved, using flow cytometry, that the separated cells had the MSC-specific markers CD90.1, CD29, but lacked CD45 and CD31. While the results of the validation of osteogenic differentiation demonstrated that MSCs could differentiate into osteocytes as indicated by the red calcium deposits in the MSCs population using Alizarin Red staining, the results of the validation of the morphology of the MSC culture were obtained as an image of cells attached to the bottom of the flask with spindle-like cell morphology under microscopic observation (Figure 1A and B). The results of isolated MSC cells were verified using flow cytometry to demonstrate that MSCs were able to express multiple MSC surface markers, which is consistent with their osteogenic capacity. According to the validation findings, MSCs could express CD90.1 (99.80%) and CD29 (94.20%), CD45 (1.60%) and CD31 (6.60%) (Figure 1C).

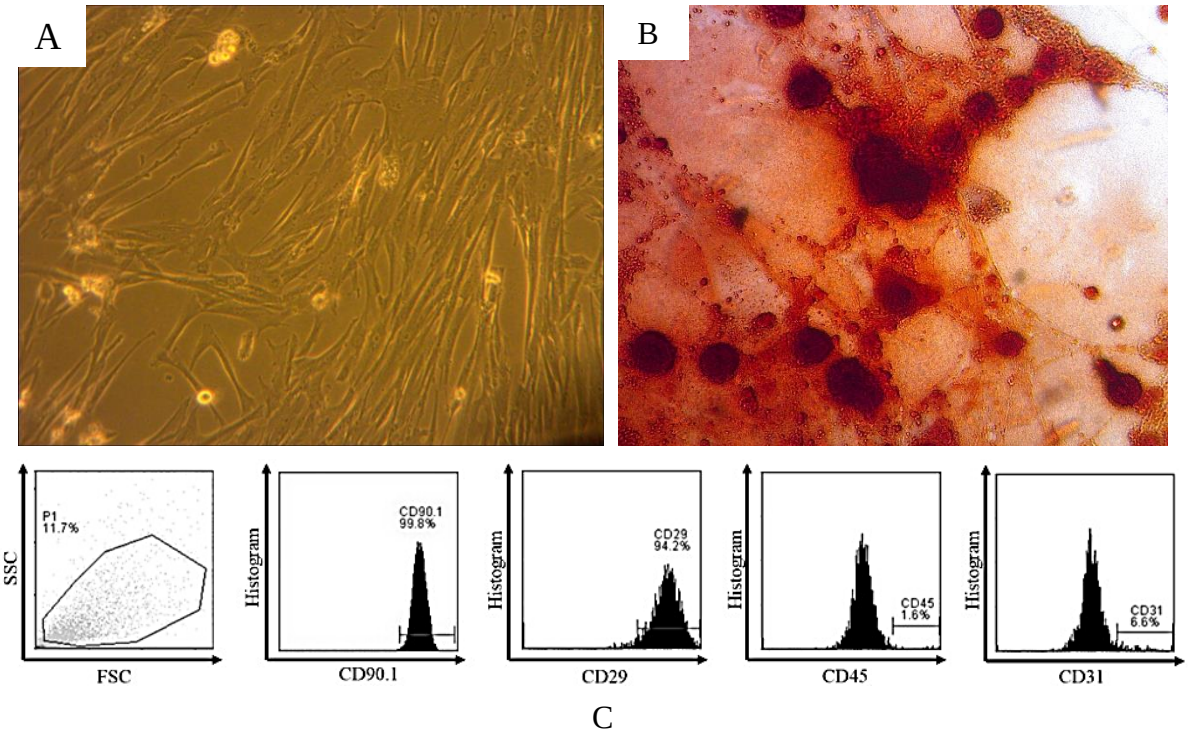

Figure 1. MSC Validation. (A) Isolated MSCs with 80% confluent showed spindle-like cells (pointed by arrows) at 100x magnification. (B) Osteogenic differentiation using Alizarin Red staining appears in the MSC population at 100x magnification. (C) Flow cytometry analysis of the expression of CD90.1, CD29, CD45, and CD31.

Secretome Profiling by ELISA

The hypoxia MSCs main secreted substances in the extracellular area are all represented by the S-HMSCs. Multiple growth factors, chemokines, cytokines, soluble proteins, free nucleic acids, lipids, and extracellular vesicles were discovered to be produced and secreted by MSCs. 0.2 m-filtered culture supernatant was collected and tested using an ELISA kit (BIOENZY, Indonesia) to identify and examine the components released by MSCs. Considering the outcome shown in Table 1.

Table 1. MSC Secretome Profiling by ELISA

Content	Mean ± SD (pg/mL)
VEGF-A	1183,01±81,23
TGF-β1	299,45±31,92
PDGF-BB	892,13±53,90

IL-10	349,21±26,04

Secretome MSCs regulate α-SMA protein expression

Based on histological analysis of rectal tissue immunohistostaining, α-SMA tissue protein were expressed as brown color under microscopical observation. According to the aforementioned findings, after hemorrhoid induction, α-SMA was expressed 20% higher than normal group. Furthermore, as was noted that the 150 µL and 300 µL of S-HMSCs significantly 15% and 20% lower than the Untreated group, respectively. In comparison to the Sham group, the effects of both dosage treatments on α-SMA expression were not significantly different. This suggests that although it is the same as normal circumstances or healthy. Image J Software was used to measure the IHC results based on the brown color area, and (Figure 2B) shows the outcomes in graphical data.

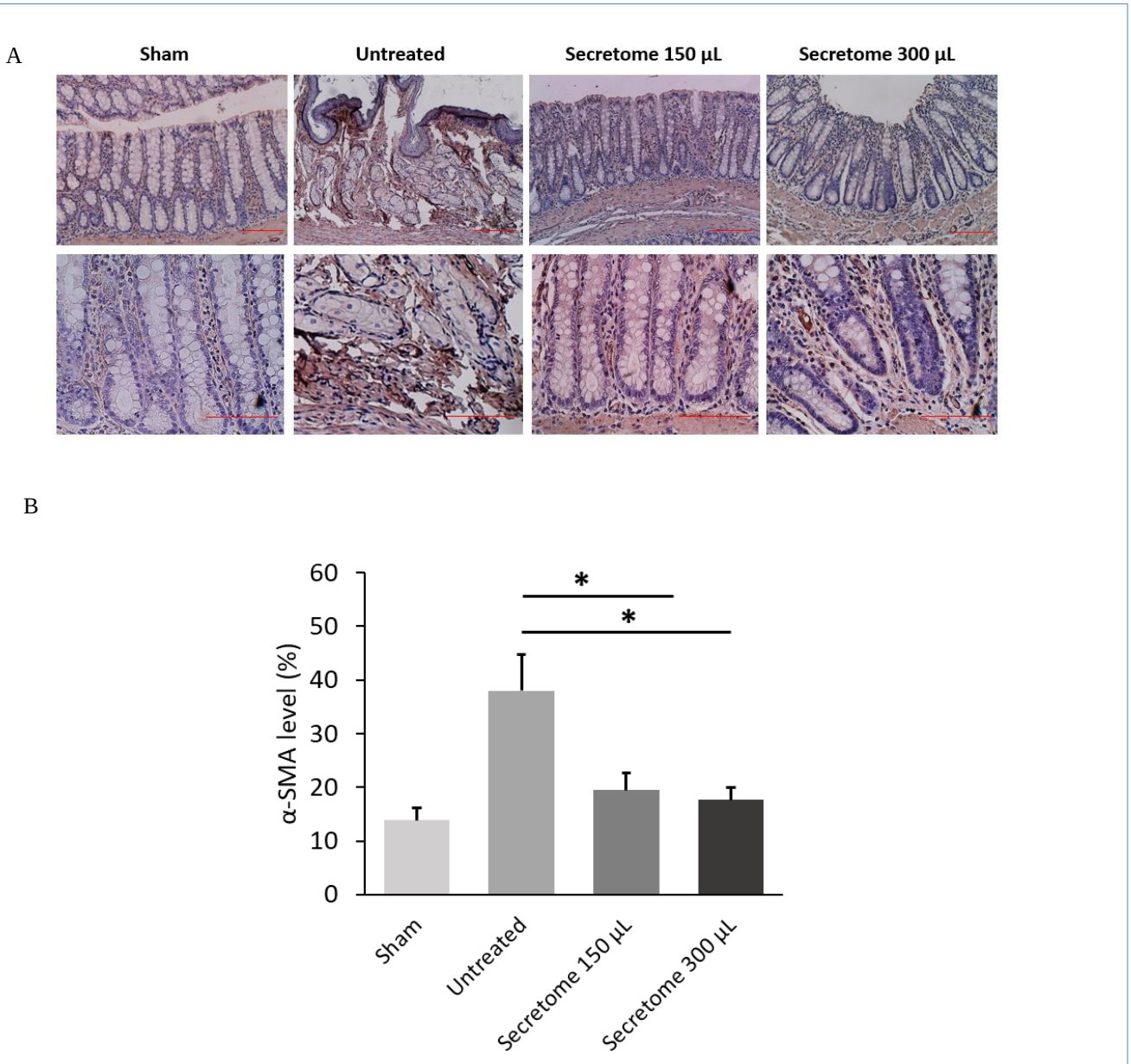

Figure 2. (A) Representative images of rectal tissue α-SMA IHC staining, the positive α-SMA express in brown color. (B) Percentage area of α-SMA histological analysis for each group. Each bar shows the mean value (standard deviation, n=5). * p<0.05. Scale bar: 100µm.

DISCUSSION

The histological analysis of rectal tissue immunohistostaining played a pivotal role in elucidating the expression of α-SMA protein within the tissue microenvironment. The visualization of α-SMA protein through brown coloration under microscopic observation provided valuable insights into the dynamic changes associated with hemorrhoid induction. Our findings demonstrated a significant increase of α-SMA expression by approximately 20% following hemorrhoid induction, as compared to the normal group. This observation aligns with the existing literature, underscoring the role of α-SMA as a marker of myofibroblast activation during tissue remodeling processes.

Intriguingly, our investigation into the therapeutic potential of S-HMSCs revealed compelling results. Both the 150 µL and 300 µL dosages of S-HMSCs exhibited a remarkable reduction in α-SMA expression levels. Specifically, the 150 µL dosage led to a 15% decrease, while the 300 µL dosage resulted in a 20% reduction compared to the Untreated group. These findings suggest that S-HMSCs could effectively modulate myofibroblast activity, thereby mitigating the heightened α-SMA expression induced by hemorrhoid formation. This aligns with previous studies highlighting the immunomodulatory and regenerative properties of MSCs Secretome, mainly are orchestrated by TGF-β, IL-10, VEGF, and PDGF[29,30]. TGF-β contained in S-HMSCs may activate fibroblast into myofibroblast that synthesis numerous ECM in order to rebuild tissue architecture[31]. Myofibroblasts, are key players for maintaining tissue homeostasis and tissue repair by communicating with their microenvironment[21]. α-SMA is the potent marker of myofibroblast.[28,32,33]

Interestingly, our investigation did not detect any statistically significant differences between the effects of the two dosage treatments compared to the Sham group. This finding suggests that the therapeutic effects either high dose or low dose S-HMSCs on α-SMA expression are comparable to the basal physiological conditions, as represented by the Sham or Healthy group. These results underscore the potential of S-HMSCs to restore α-SMA expression to levels reminiscent of healthy tissue. For quantitative analysis of the immunohistochemical staining, we employed Image J Software to measure the area of brown coloration representing α-SMA expression. The graphical data presented in Figure 2B visually encapsulates the outcomes of the immunohistochemical analysis, providing a concise representation of the observed trends in α-SMA expression across the different experimental groups.

Our study sheds light on the intricate interplay between α-SMA expression and hemorrhoid induction, while also unveiling the potential of S-HMSCs as therapeutic agents for restoring α-SMA expression to near-physiological levels. Further investigation into the underlying mechanisms driving these effects, such as paracrine signaling and immune modulation, could yield deeper insights into the therapeutic potential of S-HMSCs in the context of hemorrhoidal disease. Additionally, long-term studies assessing the durability of these effects and potential clinical applications are warranted to fully harness the translational value of these findings.

CONCLUSION

S-HMSCs may restore α-SMA protein tissue expression in croton oil-induced hemorrhoid rats.

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AUTHORS' CONTRIBUTIONS

MHBK concept and design the study and wrote the manuscript, SAH prepared and analyzed the data, and NDA contributed to revisions of the manuscript and approved it for publication.

COMPETING INTERESTS

The authors declare no competing interests.

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