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Hypoxia-preconditioned mesenchymal stem cells attenuate peritoneal adhesion through TGF-β inhibition

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ABSTRACT

BACKGROUND
Peritoneal adhesions (PAs) are generally described as fibrous bands between intra-abdominal organs following an abdominal surgical operation. The definitive treatments of PAs are currently ineffective yet. Hypoxia-mesenchymal stem cells (H-MSCs) have a higher capability to survive at the site of injury than normoxia-MSCs (N-MSCs) to repair injured tissue without fibrosis. This study aimed to analyze the effect of H-MSCs in controlling formation of PAs by reducing TGF-β level in a rat model.

METHODS
A study of post-test only control group design was conducted, involving eighteen PA rat models weighing 250 ± 25 g that were randomly assigned into 3 groups, comprising control group (C), and groups T1 and T2 receiving H-MSC treatment at doses of 3×10^6 and 1.5×10^6, respectively. To induce H-MSCs, MSCs were incubated in hypoxic conditions at 5% O_2 and 37°C for 24 hours. Expression level of TGF-β was analyzed by enzyme-linked immunosorbent assay (ELISA) at 450 nm and adhesion formation was described macroscopically. The Kruskal-Wallis variance analysis was used to analyze significant differences among the groups.

RESULTS
The results of this study showed that H-MSCs in group T1 inhibited TGF-β expression significantly on day 8 ($p<0.001$) and day 14 ($p<0.05$). Moreover, there was almost no adhesion apparent following H-MSC administration in group T1.

CONCLUSIONS
Based on this study, we conclude that H-MSCs may attenuate PA formation following inhibition of TGF-β expression in the PA rat model.

Keywords: Peritoneal adhesions, hypoxia, H-MSCs, TGF-β, rat

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INTRODUCTION

Peritoneal adhesions (PAs) are fibrous bands between two or more intra-abdominal organs or between these organs and the inner abdominal wall, due to secondarily prolonged inflammation of an abdominal surgical operation.[1] The occurrence of PAs approaches 63-97% and approximately one-third of such cases are readmitted due to complications. PAs may cause complications such as abdominal or pelvic pain, small bowel obstructions, and infertility.[2] Although the definitive treatment of PAs such as laparoscopic adhesiolysis and/or laparotomy adhesiolysis has the benefit of reducing the PAs, those treatments have the potential to induce several complications such as small bowel obstructions and the risk of PA recurrence.[3-5] Therefore, more effective and safer treatment of PAs is needed.

Mesenchymal stem cells (MSCs) are a population of pleiotropic cells with self-renewing capacity that have the ability to differentiate into various canonical mesenchymal cells, namely adipocytes, chondrocytes, and osteocytes.[6] MSCs express a number of markers, such as CD29, CD44, CD73, CD90, CD105, CD166, but are lacking in CD14, CD34, CD45, or CD11b, CD79a or CD19, and HLA class II expression.[7] A recent study reported that hypoxia offers benefits to MSCs for increasing their capability of self-renewal, proliferation, and survival gene modulation.[8] Hypoxia-mesenchymal stem cells (H-MSCs) can mimic a physiological niche in the bone marrow that potentially offers the benefits of a wound healing process. This is in line with a previous study reporting that one of the therapeutic failures of MSC administration was poor engraftment capacity and low cell survivability during the transplantation process at the hypoxia injury site.[9] This fact showed that the competence of MSCs to survive under hypoxic conditions in preventing PA formation was crucial to be explored.[10] Furthermore, H-MSCs are more robust cells in controlling inflammation than N-MSCs through increasing IL-10 production to tightly compete with TGF-β for fibroblast activation.[11]

The pathogenesis of PAs involves several inflammatory factors to stimulate the prolonged release of TGF-β leading to the activation of fibroblasts and adhesion formation.[12] A previous study revealed that MSCs cultured in hypoxia exhibit higher rates of proliferation and better retain their stem cell properties, including PAs.[13] Our previous study showed that H-MSCs can survive in injured tissues and have the capacity to reduce PA formation and suppress inflammatory conditions by releasing IL-10 as a pleiotropic anti-inflammatory cytokine.[14] However, the role of H-MSCs in decreasing TGF-β as the main cytokine of the main fibrosis mechanism in PA formation remains unclear. Therefore, to decrease TGF-β through H-MSC administration for inhibiting PA formation was crucial to be investigated. In this study, we aimed to analyze the effect of H-MSCs in controlling PA formation by reducing TGF-β levels.

METHODS

Research design

This study of post-test only control group design was carried out in the Stem Cell and Cancer Research Laboratory, Faculty of Medicine, Universitas Islam Sultan Agung (Unissula), Semarang from August-October 2018.

PA animal model

The sample size was calculated according to the Federer formula. [15] This study used eighteen Wistar albino rats which were randomly divided into 3 groups, consisting of the control group, the high dose group (T1) and the low dose group (T2) (n=6 rats per group) with 10% estimated drop-out rate. The Wistar albino rats weighing between 250 and 300 g were purchased from the animal holding unit, Faculty of Veterinary Medicine, Gadjah Mada University. Rats were acclimatized and housed in 12 h light-dark cycle standard cages at 24°C with food and water *ad libitum*. After fasting for 12 h, rats were

anesthetized by intraperitoneal administration of ketamine and xylazine (90 and 10 mg per kg body weight; respectively). The surgical procedure was performed as described previously.[14] The operations were performed under aseptic conditions and took less than 20 minutes for each rat to minimize room air tissue dying effect. The musculo- peritoneal layer was incised and opened over a length of 3 cm at the linea alba after cutaneous ventral midline incision. In order to induce PAs, the standardized surgical injuries were applied to one side of the terminal ileum (3 cm from the cecum) at a size of 0.5 × 0.5 cm on the right sidewall of the ileum by brushing with a cytobrush (Gynobrush, Langenbrink, Emmendingen, Germany) until punctuate red spots were observable as visual indicator of ileum trauma. Following the intervention, the abdominal incision was closed with 3–0 polyglactin suture and the rats were reared in battery cages for 14 days**.**

MSC isolation culture and H-MSC induction

Mesenchymal stem cells were isolated from the umbilical cord (UC) obtained from pregnant single Wistar albino rats and expanded as described previously.[8] Briefly, UCs were chopped into smaller pieces and transferred into a T25 culture flask (Corning, Tewksbury, MA, USA) containing DMEM (Gibco™ Invitrogen, NY, USA) which was supplemented with 10% FBS (Gibco™ Invitrogen, NY, USA), 1% penicillin (100 U/mL) and 0.25% streptomycin (100 μg/mL) (Gibco™ Invitrogen, NY, USA) and incubated at 37°C in 5% CO_2 and e"95% humidity. The medium was replaced every 3 days and the cells harvested after reaching 80% confluence (14 days). The 4-6th passage MSC-like cells were employed for the experiments.

To induce H-MSCs, MSCs derived from the 4th passage were incubated under 5% O2 condition in a hypoxia incubation chamber (STEMCELL Technologies, Biopolis, Singapore) for 24 h at 37°C and 5% CO2, then collected for the following experiment.

H-MSC characterization

Hypoxic-mesenchymal stem cell surface markers at the 4-6th passage were analyzed by flow cytometry analysis according to company protocols. Briefly, the cells were subsequently incubated in the darkroom with allophycocyanin (APC) mouse anti-human CD73, fluorescein isothiocyanate (FITC) mouse anti-human CD90, perCP-Cy5.5.1 mouse anti-human CD105, and phycoerythrin (PE) mouse anti-human Lin negative (CD45/CD34/CD11b/CD19/HLA-DR) antibodies. H-MSC cells were stained with MSC specific antibody for 30 minutes at 4°C, then examined and analyzed with a BD Accuri C6 Plus flow cytometer (BD Biosciences, San Jose, CA, USA).

H-MSC osteogenic differentiation assay

The H-MSCs were cultured on 24-well plates (1.5 x 10^4 cells/well density) with standard medium containing DMEM (Sigma-Aldrich, Louis St, MO), supplemented with 10% FBS (Gibco™ Invitrogen, NY, USA), 1% penicillin (100 U/mL) and 0.25% streptomycin (100 μg/mL) (Gibco™ Invitrogen, NY, USA) at 37°C, 5% CO2, and e"95% humidity. After reaching 95% confluence, the standard medium was aspirated and replaced with osteogenic differentiation medium containing Human MesenCult™ Osteogenic Differentiation Basal Medium (Stem Cell Technologies, Singapore), augmented with 20% Human MesenCult™ Osteogenic Differentiation 5X Supplement (Stem Cell Technologies, Singapore) and 1% L-Glutamine (Gibco™ Invitrogen, NY, USA). The differentiation medium was replaced every 3 days. The bone matrix was formed after 15 days and was visualized by 2% Alizarin red staining.

H-MSC administration

After the surgical procedure of PA induction, the rats were treated once with 3x10^6 cells for the T1 group and 1.5 x10^6 cells for the T2 group via submucosal injection, while the control group received the omental patch treatment.

Table 1. Nair's macroscopic adhesion grade [16]

Grade 0	No adhesion
Grade 1	Only one adhesion band between abrasion site and abdominal wall
Grade 2	Two adhesion bands between abrasion site and abdominal wall
Grade 3	More than two adhesion bands between abrasion site and abdominal wall
Grade 4	Adhesion of all viscera to the abdominal wall

ELISA assay

At days 8 and 14 post-PA surgical procedure, ELISA was used to measure the level of TGF-β released in rat peripheral blood in the treatment and control groups. The analysis was done according to the manufacturer's instructions (Fine Test, Wuhan, China) using a standard curve and was performed in five replications. The colorimetric absorbance wavelength used was 450 nm.

Macroscopic analysis

The induced PA rat animal models were euthanized using CO2 inhalation on the 14th day after treatment. The adhesion formation was observed, and categorized using Nair's Adhesion Grade (Table 1). [16]

Statistical analysis

The statistical analysis was performed using the SPSS ver. 23 (SPSS Inc., Chicago, IL, USA). Descriptive data were expressed as mean ± standard error. For intergroup analysis, the Kruskal-Wallis variance analysis was used to analyze significant differences among the groups. And Mann Whitney test to compare between groups. A p-value of less than 0.05 was considered statistically significant.

Ethical clearance

The study was approved by the Health Research Ethical Committee, Medical Faculty, Universitas Sumatera Utara, Medan, Indonesia, under No. 541/TGL/KEPK FK USU-RSUP HAM/2019.

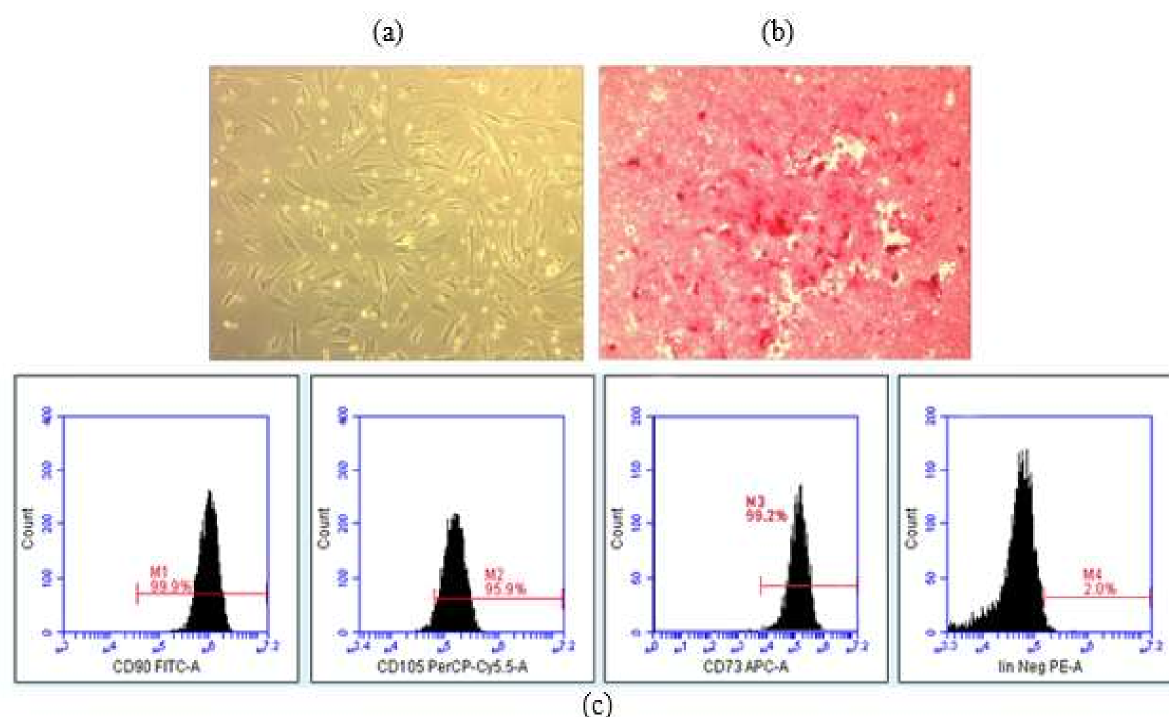


Figure 1. (a) H-MSCs from the in-vitro culture showing spindle shape and fibroblast-like characteristics (10x magnification) ; (b) Osteogenic differentiation analysis using alizarin red staining showing calcium deposition marked by red color ; (c) Flow cytometry analysis of H-MSCs confirmed that H-MSCs expressed high levels of CD90, CD105, CD73 and lacked the expression of Lin

Figure 2. Macroscopic appearance showing severe PA in Control group at grade 4 (red arrow) (a). Treatment groups showing fewer adhesions in the T2 group (b); and almost no adhesions in the T2 group (black arrow) (c)

RESULTS

H-MSC characterization and differentiation

The H-MSCs that were incubated from the 4th passage at 80% confluence under hypoxic conditions (5% O_2) showed denser fibroblast and spindle-like shape characteristics (Figure 1a). After undergoing the osteogenic differentiation assay the H-MSCs showed calcium deposition under 2% Alizarin red staining (Figure 1b). The flow cytometry analysis confirmed that H-MSCs expressed high levels of CD90 (99.9%), CD105 (95.9%), CD73 (99.2%) and lacked the expression of Lin^- (2.0%) thereby demonstrating a characteristic immunophenotype of MSCs (Figure 1c).

Macroscopic assessment

The Nair macroscopic adhesion grade was used to describe adhesion occurrence in this study. There was Grade 4 adhesion in the control group, no adhesion in group T1 (Grade 0), and less adhesion in group T2 (Grade 1) (Figure 2).

TGF-β expression analysis

We analyzed TGF-β levels as an inflammatory marker of the ileum tissue. TGF-β expression was detected using ELISA assay. The results showed that the TGF-β expression level in groups T1 and T2 were significantly lower than in the control group on both the 8th and 14th days ($p<0.05$) (Table 2).

DISCUSSION

PAs are commonly fibrous bands occurring after abdominal surgery that have severe complications of life quality.[17] The pathology of peritoneal fibrinolysis reduction is thought to play a key role in PA pathogenesis.[18] Inflammatory cytokines such as IL-17 may also promote PAs by increasing the activation of fibroblasts along with TGF-β1 stimulation.[19] A previous study reported the potential of MSCs to treat PA formation.[20] However, although MSCs were able to reduce peritoneal inflammation, they were ineffective in controlling PAs.[21] Hypoxia-MSCs have positive effects on the in vitro stemness, survival capacity, and angiogenic potential that is mediated by hypoxia-inducible factors (HIFs), such as HIF-1α and HIF-2α.[22] To analyze the effect of H-MSCs in preventing PA formation, we induced abrasion in rats as the established animal model of PAs according to a previous

Table 2. TGF-β levels according to treatment groups at 8th and 14th days

	Treatment groups			p value*
	T1	T2	C	
TGF-β level (pg/mL)				
8th day	492.04±53.50	645.05±59.89	699.68±18.75	T1 = 0.008; T2 = 0.008
14th day	450.88±20.86	539.50±43.79	563.55±26.24	T1 = 0.008; T2 = 0.032

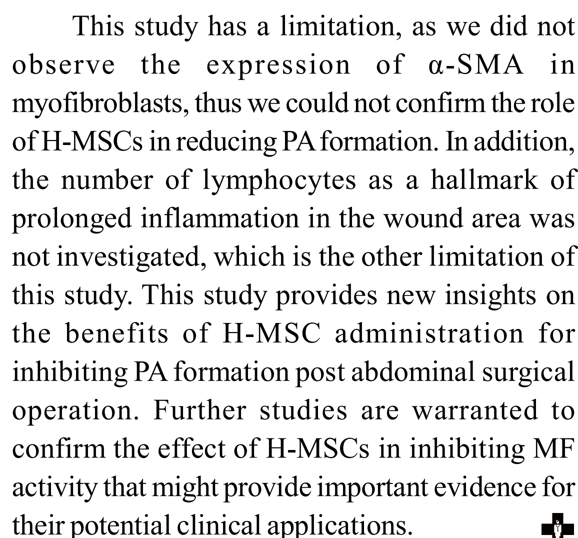
T1: H-MSC 3x10^6 cells; T2: H-MSC 1.5x10^6 cells; C: NaCl; *Kruskal-Wallis and Mann Whitney test

study.[23] In this study, we transplanted the H-MSCs into PA rat models directly to the ileum by submucosal injection, then analyzed the TGF-β on day 8 and 14.

Our study found that there was a significant decrease in TGF-β levels in the T1 and T2 groups on days 8 and 14. Our results indicate that there was complete wound healing in the H-MSC groups as compared with the control group. This finding was in line with our Nair macroscopic analysis in which there were PAs in the control group but no adhesion formation in the H-MSC treatment groups. A previous study reported that MSCs could attenuate peritoneal adhesion through increasing the level of TNF-α Stimulating Gene-6 (TSG-6) to reduce the inflammatory process.[24] This is in line with our previous study revealing that H-MSCs could increase the level of IL-10 as a major anti-inflammatory cytokine to control adhesion formation.[14] This indicated that H-MSCs could accelerate wound healing and attenuate PA formation through the decrease in TGF-β levels. A recent study found that pro-inflammatory cytokines such as TNF-α and IL-1β may stimulate TGF-β production to induce the activation of myofibroblasts (MFs) for PA formation.[25] A previous study also reported that H-MSCs could decrease the expression levels of various pro-inflammatory cytokines, such as TNF-α, IL-1β, and MIP-2, and promote the secretion of anti-inflammatory molecules, including IL-10 and prostaglandin E_2 (PGE_2).[26] The IL-10 as anti-inflammatory molecules could suppress the TGF-β level which might be attributed to the inactivation of MF that have crutial roles in adhesion formation.[27]

This study also demonstrated the dose-dependent effect of H-MSCs. This was in accordance with a previous study that found a progressive increase in the number of altered cytokines at higher doses of MSCs.[28,29] The interaction between these pro- and anti-inflammatory molecules at the injury site following H-MSC administration may explain the TGF-β lowering effect of H-MSCs. This was supported by our morphological findings, in which there were fibrous bands of PAs in the control group. These findings indicated that inflammation could be optimally controlled by high doses of H-MSCs. The fibrin matrix contained in the initial adhesion was replaced by extracellular matrix (ECM) under prolonged inflammation due to the ECM synthesis being more active than the degradation process, leading to the formation of new endothelial layers connecting to other organs.[30,31] The activated MFs that were characterized by α-Smooth Muscle Actin (α-SMA) were the main actors of these processes to produce ECM in excessive amounts due to the incompetence of IL-10 of the control group to suppress the inflammation.[14,21]

This study has a limitation, as we did not observe the expression of α-SMA in myofibroblasts, thus we could not confirm the role of H-MSCs in reducing PA formation. In addition, the number of lymphocytes as a hallmark of prolonged inflammation in the wound area was not investigated, which is the other limitation of this study. This study provides new insights on the benefits of H-MSC administration for inhibiting PA formation post abdominal surgical operation. Further studies are warranted to confirm the effect of H-MSCs in inhibiting MF activity that might provide important evidence for their potential clinical applications.



CONCLUSIONS

In conclusion, our data demonstrate that H-MSCs attenuate PA formation through suppressing TGF-β expression.

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