ORIGINAL ARTICLE

Mesenchymal stem cells under hypoxia condition inhibit peritoneal adhesion by suppressing the prolonged release of interleukin-6

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ABSTRACT

Aim To investigate the role of hypoxia-preconditioned mesenchymal stem cells (H-MSCs) in preventing peritoneal adhesion by regulating IL-6 at days 6 and 12.

Methods Twenty-four PAs rat model weighing 250 g to 300 g were randomly allocated into 4 groups: sham (Sh), control (C), H-MSCs treatment group at dose 1.5 x 10^6 (T1) and 3 x 10^6 (T2). To induce H-MSCs, all MSCs population were incubated under hypoxia state (5% O_2), 5% CO_2, and 37° C for 24 hours. Expression level of IL-6 was performed using ELISA. Morphological appearance of adhesion was observed by visualizing the existence of adhesion formation in intestinal.

Results In this study we found that there was a trend of decrease of IL-6 level on day 6 following MSCs treatments. Interestingly, there was a significant decrease of IL-6 level on day 12 in all treatment groups. Also, no adhesion occurred in T2 group.

Conclusions H-MSCs prevent PA development by suppressing the prolonged release of IL-6 at proliferation phase.

Key words: abdominal pain, abdominal surgery, cellular, inflammatory cytokines

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INTRODUCTION

Peritoneal adhesion (PA) is the fibrous adhesion that mostly occurs following abdominal surgery and prolonged intra-abdominal inflammation (1). The incidence of PA post general abdominal laparotomy ranges from 60 to 90% and reaches up to 97% post gynaecologic pelvic operation in which 10-20% of those may develop serious health problems, such as bowel obstruction that potentially increases mortality and morbidity (2,3). The inflammation is the main actor in PA formation due to the release of several cytokines, particularly IL-6 that have responsibility in maintaining the organization of fibrin gel matrix leading to PA development (4). Delayed removal of the fibrin gel matrix during remesothelialization process of wound peritoneal healing indicated inadequate activities of fibrinolytic in lysing the fibrin matrix associated with the continuous release of IL-6 (5-6). However, IL-6 also has a responsibility in promoting cellular proliferation during the intestinal wound healing (7). Therefore, to prevent PA formation, the control of inflammation using immunosuppressive agents, including mesenchymal stem cells under hypoxia condition (H-MSCs) is a crucial point to be explored.

The importance of the H-MSCs in the physiology of multipotent cellular species is responsible for tissue organogenesis, including intestinal regeneration in PA. Accordingly, the initial stage of repair at the wounds site, including PA occurs in a hypoxia milieu in which heterogeneous cell populations including MSCs are present. A previous study reported that H-MSCs have a robust ability in inhibiting PA formation than MSCs through accelerating the shift of inflammation to proliferation phase (8) leading to the acceleration of injured tissues healing (9). The hypoxia precondition employed on MSCs in vitro could generate distinctive changes in stem cell characteristics and influence the secretion of cytokines and growth factors. Moreover, mimicking the natural microenvironment by decreasing in vitro O_2 tension on MSCs could also provide the optimum capability of stem cell migration in vivo (10,11). H-MSCs as multipotent cells express the high level of specific markers such as CD29, CD44, CD73, CD90, CD105 and CD166 and lack of the expression of CD11b, CD14, CD34, CD45, or, CD79a or CD19, and HLA class II. H-MSCs also have the differentiation capacity into various specific mature cells such as adipocyte, osteocyte, chondrocyte and neurocyte (12). These immunomodulatory properties of H-MSCs are potentially employed to control proinflammatory peritoneal by attenuating IL-6, which is associated with PA development.

Peritoneal healing is initiated by the proliferation of new mesothelium in surrounding cellular matrix at the injury time up to 3 - 5 days. One major step in PA formation is inadequate fibrinolytic activity to degrade the fibrin gel matrix containing coagulation factors and inflammatory cells over the damaged peritoneal surface (13). IL-6 released by inflammatory cells actively promotes the coagulation cascade by increasing tissue factor and fibrinogen production without affecting fibrinolysis (14). However, prolonged release of IL-6 may also stimulate plasminogen activator PAI-1 and PAI-2 to inhibit fibrinolytic activity leading to PA development (15). On the other hand, the enhancement of IL-6 at the early inflammatory phase may also stimulate intestinal cell proliferation that is important for wound peritoneal healing (16). The role of H-MSCs in preventing PAs has been explored by our previous study reporting that H-MSCs could inhibit PA formation by accelerating the shift of inflammatory to proliferation phase through increasing IL-10 (17). Therefore, regulating IL-6 as one of the responsible factors in PA formation through H-MSCs administration during peritoneal healing remains to be elucidated.

The aim of this study was to analyse the role of H-MSCs in suppressing the prolonged release of IL-6 during peritoneal adhesion prevention.

MATERIAL AND METHODS

Study design and animal model

A total of 24 healthy 8-week-old male Wistar rats weighing 300 ± 30 gr (cv= 10%) were reared, acclimatized, and fed ad-libitum under 12 h light-dark photoperiod in battery cages at 24°C, 60% humidity. 12-h fasted rats were anesthetized intraperitoneally using ketamine and xylazine (90 mg/kg and 10 mg/kg body weight, respectively). Briefly, 25 mm^2 standardized injury area was applied to the right sidewall of ileum by scrubbing with a cytobrush (Gynobrush, Langenbrink, Emmendingen, Germany) until the punctuate red colour visually appeared as an indication of ile-

um trauma. The abdominal incision was sutured with 3–0 polygelatin suture and the rats were reared in battery cages for 12 days.

The study was approved by the Experimental Animal's Ethics Committee of the Medical Faculty of Universitas Islam Sultan Agung (Unissula), Semarang, Indonesia.

Methods

MSCs isolation and culture. Mesenchymal stem cells (MSCs) were isolated aseptically from the 19-day pregnant female Wistar rats' umbilical cord (RUC) by mincing into 1-2 mm-sized pieces and carefully transferred into a T25 culture flask (Corning, Tewksbury, MA, USA). The culture medium contained DMEM (Gibco Invitrogen, NY, USA), 10% FBS (GibcoInvitrogen, NY, USA), 1% penicillin (100 U/mL) and 0.25% streptomycin (100 µg/mL) (GibcoInvitrogen, NY, USA) was added and replaced every 3 days. The cultured umbilical cord (UC) was incubated under 37°C, 5% CO_2 and 95% humidity condition until the cells reached 80% confluence (14 days). The 6th passaged MSCs-like cells were used in this study.

MSCs characteristic. RUC-MSCs-like cells were confirmed by analysing MSCs specific markers and the capability to differentiate into mature cells. The 5th passage of RUC-MSCs-like was stained with fluorescence-labelled specific MSCs antibody including FITC mouse anti-rat CD45 (Clone OX-1, 554877, BD Biosciences, CA, USA), PE mouse anti-rat CD31 (Clone TLD-3A12, 555027, BD Biosciences) PerCP mouse anti rat-CD90.1 (Clone OX-7, 557266, BD Biosciences) and Alexa flour 647 hamster anti-rat CD29 (Clone Ha2/5, 562153, BD Biosciences) and examined using a BD C6 Plus flow cytometer (BD Biosciences) and BD FACSDivaTM software (BD Biosciences). In accordance with the International Society of Cellular Therapy (ISCT) (12) the MSCs have specific marker profile, such as CD90 and CD29 and negative of Lineage marker (CD45/CD34/CD31/CD11b/CD19/HLA-DR).

MSCs osteogenic differentiation. The capability of RUC-MSCs-like cells to differentiate into mature cell was confirmed using osteogenic differentiation. The RUC-MSCs-like cells at the 5th passage were incubated in osteogenic differentiation medium contained DMEM (Sigma-Aldrich, Louis St, MO), 10% FBS (Gibco Invitrogen, NY, USA), 10-7 mol/L/ 0.1 µM dexamethasone, 10 mmol/L β glycerophosphate, and 50 µmol/L ascorbate-2-phosphate (Sigma-Aldrich, Louis St, MO) under 5% CO_2, 37°C temperature, and 95% humidity until the calcium deposition was formed that could be visualized after alizarin red staining (Sigma-Aldrich Corp., St. Louis, MO, USA).

H-MSCs preparation and administration. The 6th passage RUC-MSCs were incubated in the hypoxic chamber under 5% O2, 37°C temperature, and 95% humidity for 24 h, then collected and diluted in 0.5 mL NaCl for the following experiment. The induced PAs rats were randomly divided into 4 groups: sham (S) received no healing agent, control (C) received NaCl injection around the abrasion site, T1 and T2 respectively received $1.5x10^6$ and 3 $x10^6$ HMSCs injection around the abrasion site.

IL-6 level analysis. The blood samples from 6 and 12 days after the treatment were collected from vena orbitalis and centrifuged at 3000×g for 10 minutes to obtain the serum. All samples were stored at –80°C until analysis. IL-6 level was analysed using an enzyme-linked immunosorbent assay (ELISA) plate reader (Bio-Rad, Berkeley, California, US) and IL-6 ELISA kit (Wuhan Fine Biotech Co., Ltd, Wuhan, China).

Macroscopic analysis. The induced PAs rat's animal model was euthanized using CO_2 inhalation in the 12th day after the treatment. The adhesion formation was observed, categorized using Nair's Adhesion Grade (18). Macroscopic grading was assessed by two general surgeons using the modification tool of Leach grade and Nair grade. The Nair's grade was divided into four grades according to the adhesion: 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, and grade 4 - the highest grade is marked with adhesion of all viscera to the abdominal wall.

Statistical analysis

Descriptive data were expressed in mean±standard error. For intergroup analysis, the Kruskal-Wallis variance analysis was used to analyse significant differences among the groups. A $p<0.05$ was considered statistically significant.

RESULTS

The UC-MSCs expressed CD90.1 (53.8±0.92 %) and CD29 (97.1±0.87 %), and lacked the expression of CD31 (4.73±1.62 %) and CD45 (1.73±0.38 %) (Figure 1). Furthermore, MSCs showed fibroblast-like with spindle shape characteristic and became 80% confluent after 5-7 days culture (Figure 2A), and osteogenic differentiation analysis confirmed calcium deposition appearance following bright red colour after alizarin red staining (Figure 2B).

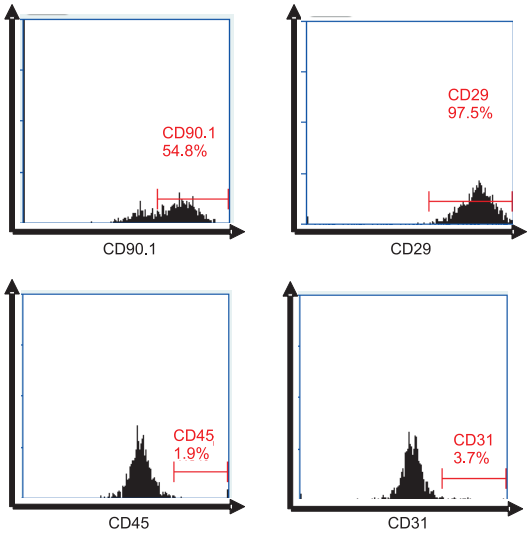

Figure 1. Detection of UC-MSCs by flow cytometry demonstrates positive expression of CD 90.1, CD29, and negative expression of CD31 and CD45

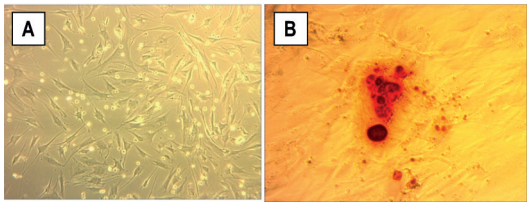

Figure 2. Morphology and differentiation of cultured mesenchymal stem cells. A) Umbilical cord candidate mesenchymal stem cells (MSCs) from in-vitro culture after 24h hypoxia; B) Osteogenic differentiation with alizarin red staining

To determine the ability of MSCs in suppressing prolonged release of IL-6 associated with PA development, we assessed the level of IL-6 in proliferation and remodelling phases using ELISA on day 6 and 12. A trend of decrease of IL-6 level on day 6 following MSCs treatments was found. Interestingly, there was a significant decrease of IL-6 level on day 12 in all treatment groups ($p<0.05$), in which T2 group showed the optimum decrease of IL-6 level (6.82 ± 5.86 pg/mL) (Figure 3).

To evaluate the PAs adhesion formation, a necropsy was performed by opening the abdominal cavity through a reverse U-shape incision on day

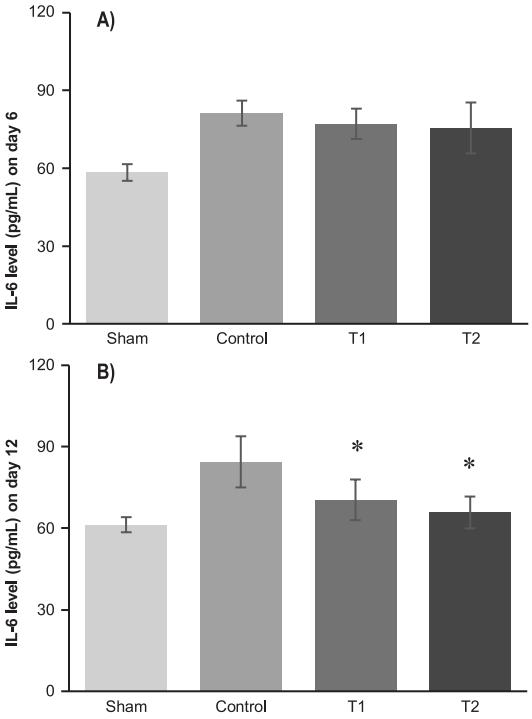

Figure 3. ELISA assay shows the significant increase of IL-6 level on T2 at day 6 (A), which became significantly decreasing following 12 days after the treatment (B ($p<0.05$)

12 following abdominal surgery. The Nair's macroscopic adhesion grade was shown. There was adhesion grade 4 in the control group, while T1 showed adhesion grade 1. Moreover, there was no adhesion band in T2 (Grade 0) (Figure 4).

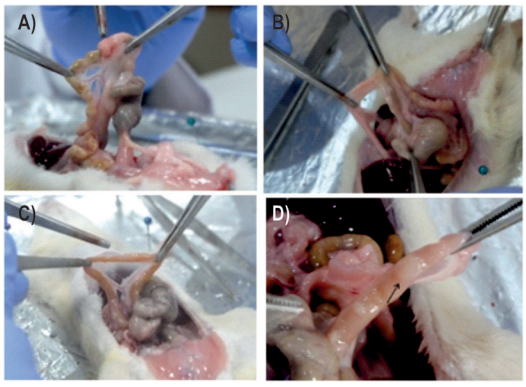

Figure 4. Peritoneal adhesion PAs were shown in the control group (A) (black arrow). There was grade 1 adhesion on T1 (B), while no adhesion was shown in the T2 group (C) (red arrow), which was similar to the sham group (D) (black arrow)

Table 1. IL-6 level after 6- and 12-days mesenchymal stem cells (MSCs) treatment in peritoneal adhesion (Pas) rat model

Group	IL-6 Level	
	Day 6 (pg/mL)	Day 12 (pg/mL)
Sham	58.36 ± 3.22	61.32 ± 2.78
Control	81.25 ± 4.84	84.46 ± 9.41
T1	77.14 ± 5.85	70.48 ± 7.48
T2	75.56 ± 9.81	65.82 ± 5.86

DISCUSSION

A study regarding the role of H-MSCs in preventing PA formation had previously reported a result in effectively controlled adhesion through suppressing inflammation at the sites of surgical (17,19) and modulating regulatory T cells (Treg) (20). However, the role of H-MSCs in regulating the inflammatory milieu-released IL-6 as the main initiator cytokines in PA formation has not been assessed. This is the first study that examines the H-MSC capabilities to control the release of IL-6 during wound peritoneal healing particularly under the proliferation and remodelling phases. The mechanisms of peritoneal healing differ from that of skin injury in which the new mesothelium developing from islands of mesothelial cells proliferate simultaneously into sheets of cells not from peripheries to the centre of injured areas as represented by the reepithelialisation of skin healing (21).

Therefore, no time differentiation in peritoneal healing between the larger peritoneal wounds and small peritoneal wounds was due to all of the remesothelialization process covering injured peritoneum within 5–6 days. Thus, to generate the PA animal model we scrubbed the right sidewall of the ileum using a cytobrush to result in prolonged inflammation that potentially triggers PA development as described by a previous study (22).

In this study, the decrease of IL-6 level in the treatment groups indicates that H-MSC administration may robustly control the inflammation by decreasing pro-inflammatory cytokines involving the IL-6 levels. The competence of H-MSC in engrafting into the injury site and suppressing the inflammatory milieu has been shown by several studies (23). Under controlled IL-6 level following H-MSC administration in those healing phases correlated with the optimum fibrinolysis activities in lysing the organization of a fibrin gel matrix leading to inhibition of PA formation (24). The activation of tissue plasminogen activator (tPA) and urokinase plasminogen activator in surgical sites induce the plasmin to degrade fibrin matrix as the main initiator of PA formation (25). This finding is in line with another study that reported the accumulation of H-MSCs in an injury site may accelerate the mesothelial proliferation that was necessary for effective wound intestinal healing (17).

Our results showed an increasing trend of IL-6 in the control group starting on day 6 and continuous increase significantly on day 12 indicating that the peritoneal healing processes were not accomplished yet. Prolonged release of IL-6 may induce the activation of PAI1 and PAI2 as fibrinolytic inhibitor triggering PA formation (15). This finding is consistent with previous studies which reported that the delayed fibrin gel matrix removal during the remesothelialization process of wound peritoneal healing potentially induces PA development (25). In line with the increase of IL-6 in the control group, the Nair's macroscopic of PA also obviously occurred in grade 3-4 adhesion. Another study revealed that IL-6 is one of the chemoattractant factors to mobilize the endogenous MSCs to specific injury in addition to leucocyte cells (27). The IL-6-induced endothelial cells express E selectin that has an important role in wound healing particularly for attracting stromal cells including mesothelial cell and MSCs (28). These findings suggested that H-MSC can optimally control inflammation by suppressing IL-6, however, the H-MSCs also simultaneously stimulate the proliferation of intestinal cell injury in an early inflammatory phase in the group treated by H-MSCs. This indicated that H-MSCs have robust ability to control the inflammatory milieu.

Furthermore, in terms of stimulating proliferation, the existence of IL-6 in early inflammation was a critical point due to the IL-6, it also has a role in stimulating epithelial intestinal proliferation (26).

This study has some limitations, in which the IL-6 in early inflammatory phase was not investigated. Hence, we have no clear observation of the role of IL-6 as a stimulating factor for proliferation of peritoneal injury. We did not analyse the PAI-1 and PAI-2, as well as tPA as the critical molecule controlled by IL-6 associated with PA development. Therefore, the association between IL-6 and PAI-1, PAI-2 and tPA still remains unclear.

In conclusion, our study showed H-MSCs could attenuate the IL-6 level in PA through significantly decreasing IL-6 level on day 12. The research was conducted based on the lack of research on the capability of MSCs as a potential immunomodulator in preventing PA development by suppressing the prolonged release of IL-6 at a proliferation phase. Therefore, this study could provide beneficial knowledge for the development of PA patients.

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TRANSPARENCY DECLARATION

Conflict of interests: None to declare.