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Mesenchymal stem cells suppress inflammation by downregulating interleukin-6 expression in intestinal perforation animal model

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Introduction: Intestinal perforation has significant fatality due to sepsis contamination and prolonged inflammation. Studies showed that mesenchymal stem cells (MSCs) secreted cytokines and growth factors to reduce inflammation. This study aims to reveal the role of MSCs in controlling inflammation in intestinal perforation wound healing by measuring interleukin-6 (IL-6) and leukocytes in injured tissue.

Materials and methods: A total of 48 rat models with a 10-mm longitudinal incision at the small intestine were divided into four groups: sham, control, Treatment group 1 (T1) injected with MSC doses of 1.5×10^6 cells and Treatment group 2 (T2) with 3×10^6 cells. IL-6 expressions were determined using western blot analysis, whereas the leukocyte infiltrations were assessed using the histopathological examination. All variables were evaluated on day 3 and 7.

Results: Leukocyte infiltration is significantly lower in T1 and T2 compared to control group in day 3 and 7 ($P < 0.05$), while there were no differences between the two treatment groups. The expression of IL-6 was found to be significantly lower in the T1 and T2 groups compared to the control group on days 3 and 7 ($P < 0.05$), with no significant differences observed between the two treatment groups.

Conclusion: MSCs administration in rats with intestinal perforation reduced inflammation by controlling leukocyte infiltration and IL-6 expression.

Keywords: IL-6, inflammation, intestinal perforation, mesenchymal stem cells

Introduction

Inflammation plays a crucial role in the early phase of wound healing. It helps remove bacteria contamination and creates a suitable tissue repair and regeneration environment. Leukocytes infiltrate and secrete cytokines and growth factors, which recruit more leukocytes into the wound site and induce wound healing. However, prolonged inflammatory response impaired wound healing, especially in intestinal perforation[1]. It might result in leakage and reperforation, which leads to an increase in mortality and morbidity rate[2].

Interleukin-6 (IL-6) is a pro-inflammatory cytokine that controls the healing process, particularly in skin wounds, by regulating the migration, proliferation, and differentiation of stem cells. This regulatory function of IL-6 underscores its significance in balancing the inflammatory response to prevent excessive inflammation, which can delay healing or lead to chronic wounds. MSCs contribute to this balance by secreting factors like IL-6, IL-8, and VEGF, which are proposed to limit inflammatory responses, thereby facilitating a more controlled and effective healing environment. By modulating the levels of IL-6, MSCs help orchestrate a conducive healing milieu, ensuring that inflammation is kept in check while allowing the necessary cellular activities for tissue repair to proceed optimally[3].

HIGHLIGHTS

- Leukocyte infiltration was significantly lower in treatment groups compared to the control group on day 3 and further decreased on day 7.
- IL-6 expressions were significantly lower in treatment groups compared to the control group on day 3 and further decreased on day 7.
- The optimum dose of MSC for the treatment of duodenal perforation is 1.5 × 106 cells/10 mm.
- MSCs administration reduces duodenal perforation inflammation by reducing leukocyte infiltration and IL-6 expression.

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Sponsorships or competing interests that may be relevant to content are disclosed at the end of this article.

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One hallmark of mesenchymal stem cells (MSCs) is the ability to control the inflammation phase through several mechanisms. MSCs can suppress the pro-inflammatory activity of myeloid cells such as macrophages, granulocytes, and monocytes. They can switch pro-inflammatory macrophages (M1) into anti-inflammatory macrophages (M2), which results in an optimal wound healing process[4]. Moreover, MSCs can also produce growth factors and cytokines to accelerate the inflammation phase. These properties help to optimize wound healing[5,6].

Extensive research has been conducted regarding the utilization of MSCs in the treatment of intestinal organs. However, there remains a notable absence of research concerning the efficacy of administering MSCs for the purpose of healing duodenal perforation wounds. This study aims to investigate the role of MSCs in controlling inflammation of duodenal perforation wound healing.

Materials and methods

MSCs isolation and culture

Umbilical cord MSCs were isolated from pregnant Wistar rat's umbilical cord. Samples were collected in a sterile culture dish with 0,9% NaCl. The umbilical cords were washed with phosphate-buffered saline (PBS) and separated from their attachment. They were mechanically minced, and blood vessels were removed. Each sample was cultured in 25T flasks containing Dulbecco's modified Eagle's medium (DMEM), fungizon, penicillin/streptomycin, and 10% fetal bovine serum (FBS) for 3 min. The tissues were incubated in a culture dish at 37°C and 5% CO_2 and fed by replacing the medium twice weekly. MSCs will emerge in ~14 days. When fibroblasts reached 80% confluence, they were detached using BDTM accutaseTM cell detachment solution (cat No. 561527).

Research design

The research utilized a posttest-only control group design to facilitate the measurement of the treatment's impact in the experimental group by comparing it with the control group, ensuring that pre-treatment conditions were equivalent between the two groups (Fig. 1). Non-probability sampling methods, specifically consecutive sampling, were employed in the sampling process, and a comparison control was established. The two pathologist performing histological assesment in this study are blinded. The research has been reported in accordance with the ARRIVE criteria[7].

Ethical clearance

Ethical approval has been given from the Commission of Medical Research Bioethics, Faculty of Medicine, Sultan Agung Islamic University with reference number: No.150/V/2021/Komisi Bioetik.

Animal model

The Ethics Committee approved all animal experimental protocols. Forty-eight male Wistar rats weighing 250–300 g were acclimated in polypropylene cages in a standard room maintained at 23–35°C with 12 light-dark cycles and 40–70% humidity for three days. The animals had free access to AIN 76A standard food and water. After fasting for 12 h, rats were anesthetized by intraperitoneal injection of 80 mg/kg of ketamine. Midline abdominal incisions were performed to expose the duodenum. A 10-mm longitudinal incision was made at the first part of the duodenum; then, the duodenal perforation was disinfected and closed by a 6/0 polypropylene (Ethicon) nonabsorbable suture at 2-mm intervals.

MSCs administration

The 48 adult male rats were randomly assigned into four groups, with six animals in each group (based on calculations using the Federer formula). The Sham group received no treatment and intervention, only sutured on the incision's closure. The control group received a local injection of 300 ul NaCl. The MSCs group received a local injection of umbilical cord MSCs at doses of $1.5x10^6$ (T1) and 3×10^6 (T2).

Animal termination

A total of 24 rats, 6 from each groups (Sham, Control, T1, T2) were terminated on day 3, and the remaining 24 rats, 6 from each groups (Sham, Control, T1, T2) were terminated on day 7 using a cocktail (Ketamine 50 mg/kg, Xylazine 10 mg/kg, and Acepromazine 2 mg/kg). Duodenums were harvested using the en bloc technique and cut into two parts. The first parts were stored in a cryotube with no RNAase at -80°C in RNA later for protein isolation; the later parts were fixated using neutral buffered formalin for a histopathology examination.

Histological assessment

Paraffin-embedded samples were cut into 4-μm sections and stained with hematoxylin and eosin according to established protocols as follows:

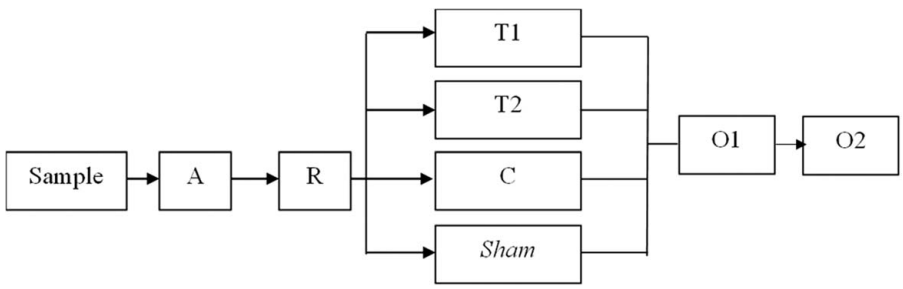

Figure 1. Research Design. A: adaptation period; R: randomization; T1: treatment group 1 [mesenchymal stem cells (MSCs) injection of 1.5 ×106 cell); T2: treatment group 2 (MSCs injection of 3 ×106 cell); C : control group; S: Sham (healthy rat); O1: first observation (day 3); O2: second observation (day 7).

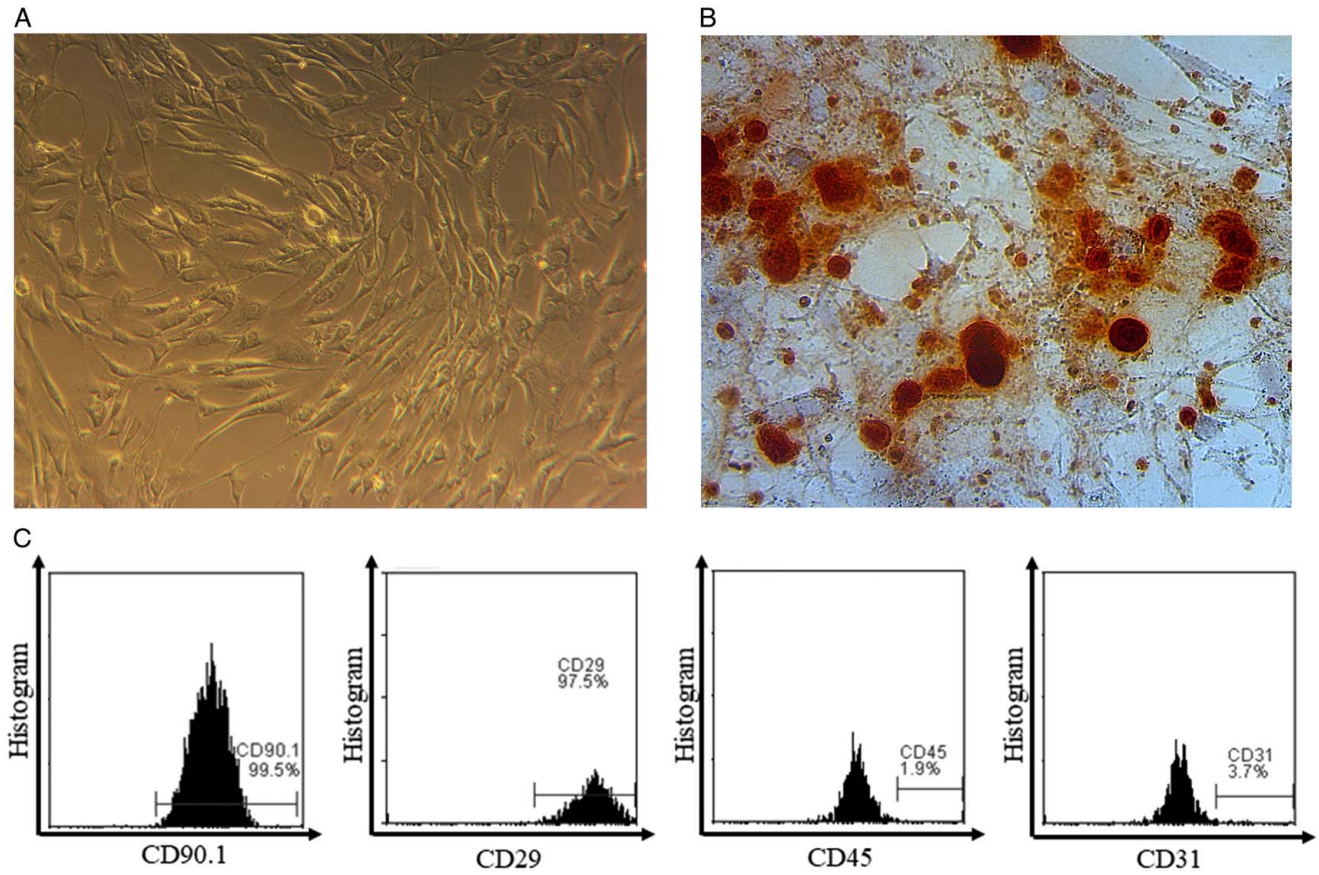

Figure 2. (A) Mesenchymal stem cells (MSCs) morphology with 90% confluency showed spindle-like cells (200 × magnification); (B) MSCs differentiation test using adipogenic culture media (200 × magnification), the red staining represents the result of post-cultured calcium deposits with osteogenic culture media; (C) Flowcytometry analysis of CD90, CD29, CD45, and CD31 expression.

(a) The specimen was placed in a glass container and immersed in xylol I solution for 2 min, followed by xylol II for an additional 2 min.
(b) It was then submerged in absolute alcohol I for 2 min, followed by absolute alcohol II for another 2 min.
(c) The specimen underwent a 2-min immersion in 95% alcohol, followed by 80% alcohol, and was then rinsed with running water.
(d) After staining with hematoxylin for 10 min and eosin for 2–3 min, the specimen was dried.
(e) The specimen was sequentially dipped in 95% alcohol for 10 times, followed by absolute alcohol for 10 dips, and finally soaked in absolute alcohol II for 2 min.
(f) The specimen underwent a 1-min soak in xylol I, followed by a 2-min soak in xylol II.
(g) Permount adhesive was applied to the specimen and covered with a cover slip.

The painted preparations were then recorded using an Optilab Advance Plus camera with Optilabviewer 4.0 software connected to an Olympus CX22 microscope for each preparation. Histological assessment was conducted by two independent blinded pathologists. The quantification of leukocyte infiltration was conducted under a light microscope with 400 × magnification. The number of leukocytes counted included lymphocytes, monocytes, neutrophils, and basophils.

Western blotting

IL-6 expressions were determined using a western blot. The lysate is made using extracted tissue, and the sample preparation solu-

Table 1

Normality and homogenity test for the degree of inflamation on day 3

	Sham	Control	T1	T2
Mean ± SD (%)	22.50 ± 2.51	53.17 ± 5.30	30.50 ± 6.02	31.25 ± 5.13
Shapiro–Wilk	0.264	0.920	0.586	0.317
Levene test		0.304		

Table 2

ANOVA and post hoc test for degree of inflammation on day 3

	Sham	Control	T1
Anova	0.001*		
Post hoc			
Control	0.001*		
T1	0.011*	0.001*	
T2	0.006*	0.001*	0.795

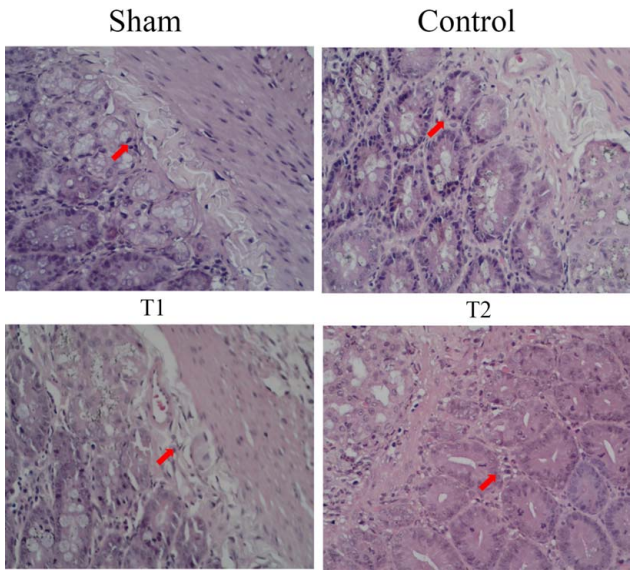

Figure 3. Histopathology of duodenal tissue of various study groups using hematoxylin and eosin staining showing leukocytes infiltration on day 3 (400× magnification).

tion (RIPA, PMSF, NaF, and cocktail inhibitor protease) is added and centrifuged at 4°C for 10 min. The obtained supernatants were used to quantify the protein using 260 nm and 280 nm wavelength UV-Vis spectrophotometry consecutively. The volume was measured and loaded into each well. Electrophoresis was performed by transferring each lysate into an Eppendorf and adding a 10× loading buffer. The hardened gel was installed into each chamber, and an SDS buffer was added. The sample and prestained marker were then loaded into an electrophoresis kit and transferred into a PVDF buffer container. PVDF membranes were soaked with methanol and, transferred into the container, and run for 1 hour. Then, the membrane was transferred into a blocking buffer and incubated for 1 hour. Anti-IL-6 antibody (SC-1351, Santa Cruz Biotechnology) was added and incubated at 4°C, then washed three times, then the secondary antibody was added and incubated for 1 h. Visual detection was performed using a luminograph(Atto).

Statistical analysis

Statistical analyses were conducted to determine the mean, mode, and median of the data. The normality of the data was assessed using the Shapiro–Wilk test due to the sample size being less than 50, while the homogeneity was tested using the Levene Statistics test. If the data were found to be normally distributed and homogeneous, the one-way ANOVA test was utilized to assess differences among treatment groups. In cases where the data was not normally distributed, transformations were applied for confirmation, with the Kruskal–Wallis test being used if normality could not be achieved. Post hoc analysis was conducted using the Bonferroni test on normally distributed data. A *P* value of less than 0.05 was considered significant. Data processing and analysis were performed using SPSS 25.0 for Windows (IBM Corp.).

Table 3

Normality and homogenity test for the degree of inflamation on day 7

	Sham	Control	T1	T2
Mean ± SD (%)	25.00 ± 2.81	42.92 ± 7.40	33.00 ± 5.20	27.50 ± 4.17
Shapiro–Wilk	0.265	0.862	0.625	0.378
Levene test		0.220		

Table 4

ANOVA and post hoc test for degree of inflammation on day 7

	Sham	Control	T1
Anova	0.001*		
Post hoc			
Control	0.001*		
T1	0.011*	0.001*	
T2	0.006*	0.001*	0.795

Results

Differentiation and characteristics of MSCs

MSCs were isolated from the umbilical cords of 21-day pregnant female rats. The isolation results were then cultured on a standard medium at 37°C and 5% C. MSCs culture after the fourth passage showed adhered cells on the bottom of the flask recognized as fibroblast-like cells (Fig. 2). Standard osteogenic and adipogenic assays were used to assess their differentiation ability. The validation of MSCs markers was carried out using flow cytometric analysis, which revealed that MSCs were positive for CD90 (99.8%) and CD29 (94.2%). In contrast, there were negatives for CD45 (1.8%) and CD31 (6.6%) expressions.

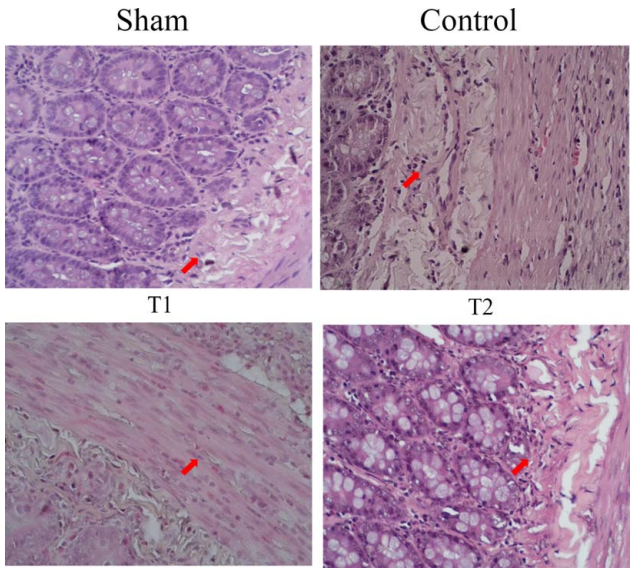

Figure 4. Histopathology of duodenal tissue of various study groups using hematoxylin and eosin staining showing leukocytes infiltration on day 7 (400× magnification).

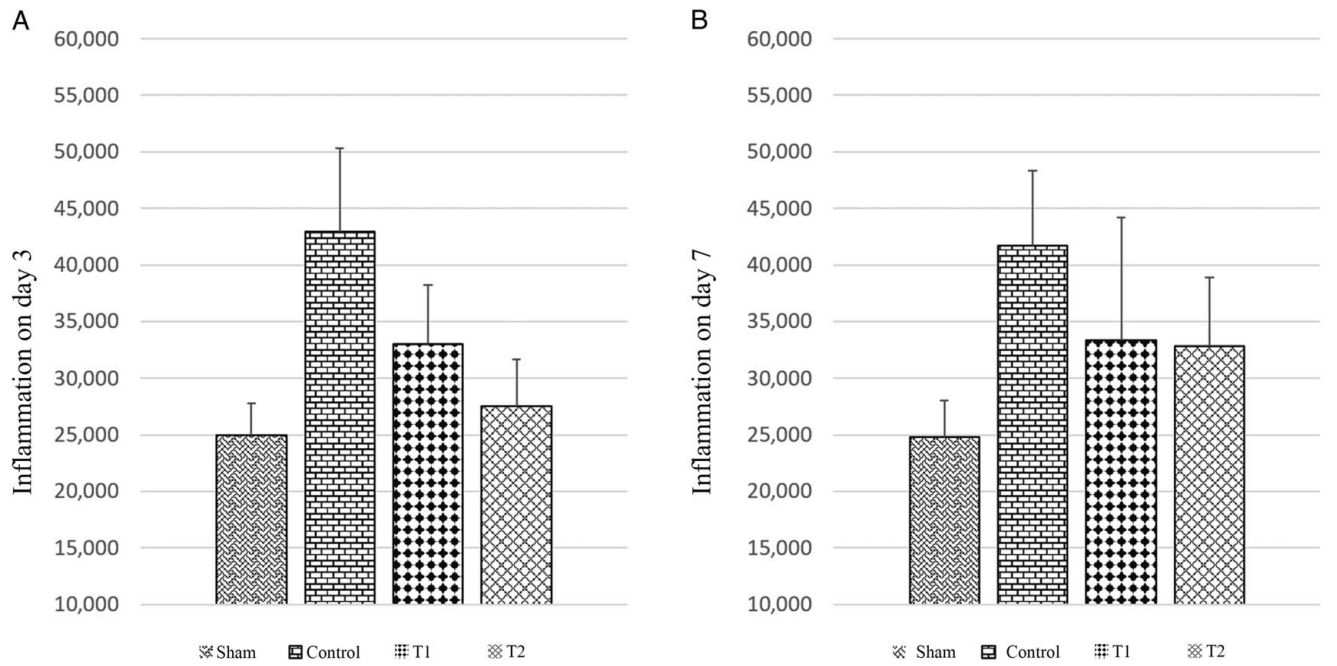

Figure 5. Quantification of leukocytes infiltration in various study groups, T1 and T2 had a significantly lower number of leukocytes compared to control group ($P = 0.001$).

Leukocytes infiltration

The degree of duodenal inflammation on day 3 was lowest in the sham group and highest in the control group. Table 1 shows that the Shapiro–Wilk and Levene tests show normal and homogeneous data *P* greater than 0.05, so it can be continued with the ANOVA and post hoc tests. ANOVA test on day 3, found a significant difference between groups ($P = 0.001$), while the Post Hoc test found a significant difference ($P = 0.001$) between T1 (30.50 ± 6.02) and T2 (31.25 ± 5.13) groups and the control group(53.17 ± 5.30). However, there was no significant difference between T1 and T2 groups ($P = 0.795$) (Table 2) (Fig. 3).

Descriptive data on the degree of inflammation on day 7 showed the lowest inflammation is found in the sham group (25.00 ± 2.81) and the highest in the control group (42.92 ± 7.40). The Shapiro–Wilk and Levene tests showed normal and homogeneous data ($P > 0.05$) (Table 3), so it could be continued with parametric tests. The results of the ANOVA test on day 7 showed significant differences between groups ($P = 0.001$). In the post hoc test, groups T1 and T2 were significantly different from the control group (Control: 42.92 ± 7.40)(T1: 33.00 ± 5.20; $P = 0.003$)(T2: 27.50 ± 4.17; $P = 0.001$), while T1 and T2 groups did not show significant differences ($P = 0.080$) (Table 4) (Fig. 4) (Fig. 5).

Table 5

IL-6 normality and homogenity test on day 3

	Sham	Control	T1	T2
Mean ± SD (%)	5.03 ± 0.08	3.38 ± 0.06	1.33 ± 0.06	1.04 ± 0.38
Shapiro–Wilk	0.120	0.647	0.483	0.761
Levene test		0.120		

IL-6 expression

IL-6 expression on day 3 was lowest in the T2 group (1.04 ± 0.38) and highest in the sham group (5.03 ± 0.08). The Shapiro–Wilk and Levene tests obtained normal and homogeneous data ($P > 0.05$)(Table 5), so that it could be continued with parametric tests. IL-6 ANOVA test on day 3 obtained significant values between groups ($P = 0.001$). Post hoc test showed that groups T1 and T2 gave significant results compared to the control group (3.38 ± 0.06)(T1: 1.33 ± 0.06; $P = 0.001$) (T2: 1.04 ± 0.38; $P = 0.001$), while there were no differences between the two treatment groups ($P = 0.113$)(Table 6).

On day 7 (Table 7), IL-6 expression was lowest in the sham group (0.15 ± 0.01) and highest in the control group (2.21 ± 0.04). The Shapiro–Wilk and Levene tests obtained normal and homogeneous data ($P > 0.05$) so that it could be continued with parametric tests. ANOVA test shows significant differences between groups on day 7 ($P = 0.001$). The Post Hoc test found a significant difference between groups T1 and T2 against the control group (Control: 2.21 ± 0.04) (T1: 1.03 ± 0.04; $P = 0.003$) (T2: 0.88 ± 0.05; $P = 0.001$). T1 compared to T2 group was found to be insignificant ($P = 0.080$) (Table 8)(Fig. 6).

Table 6

ANOVA and post hoc test for IL-6 test on day 3

	Sham	Control	T1
Anova	0.001*		
Post hoc			
Control	0.001*		
T1	0.001*	0.001*	
T2	0.001*	0.001*	0.113

IL-6, interleukin-6.

Table 7

IL-6 normality and homogenity test on day 7

	Sham	Control	T1	T2
Mean ± SD (%)	0.15 ± 0.01	2.21 ± 0.04	1.03 ± 0.04	0.88 ± 0.05
Shapiro–Wilk	0.075	0.857	0.589	0.172
Levene test		0.072		

Table 8

ANOVA and post hoc test for IL-6 test on day 7

	Sham	Control	T1
Anova	0.001*		
Post hoc			
Control	0.001*		
T1	0.014*	0.003*	
T2	0.412	0.001*	0.080

IL-6, interleukin-6.

Discussion

Duodenal perforation wound healing

Duodenal perforation is known as a rare but potentially lethal gastrointestinal emergency with a mortality rate ranging from 4 to 30%[8–10]. The management of duodenal perforation can be challenging due to its high reperforation and complication rate. The duodenum is considered to be minor and has a thinner wall than other gastrointestinal hollow organs, leading to weaker wound closure when perforation happens[11].

During the wound healing process, the inflammation phase is essential for hemostasis, recruitment of innate responses, and clearing of debris. During the inflammation phase, macrophages modulate the immune response by producing some cytokines and growth factors. Uncontrolled inflammation could lead to a poor repair process. A study reported by Qian *et al.*[1] showed a direct link between prolonged inflammation, delayed wound closure, and increased tissue scarring.

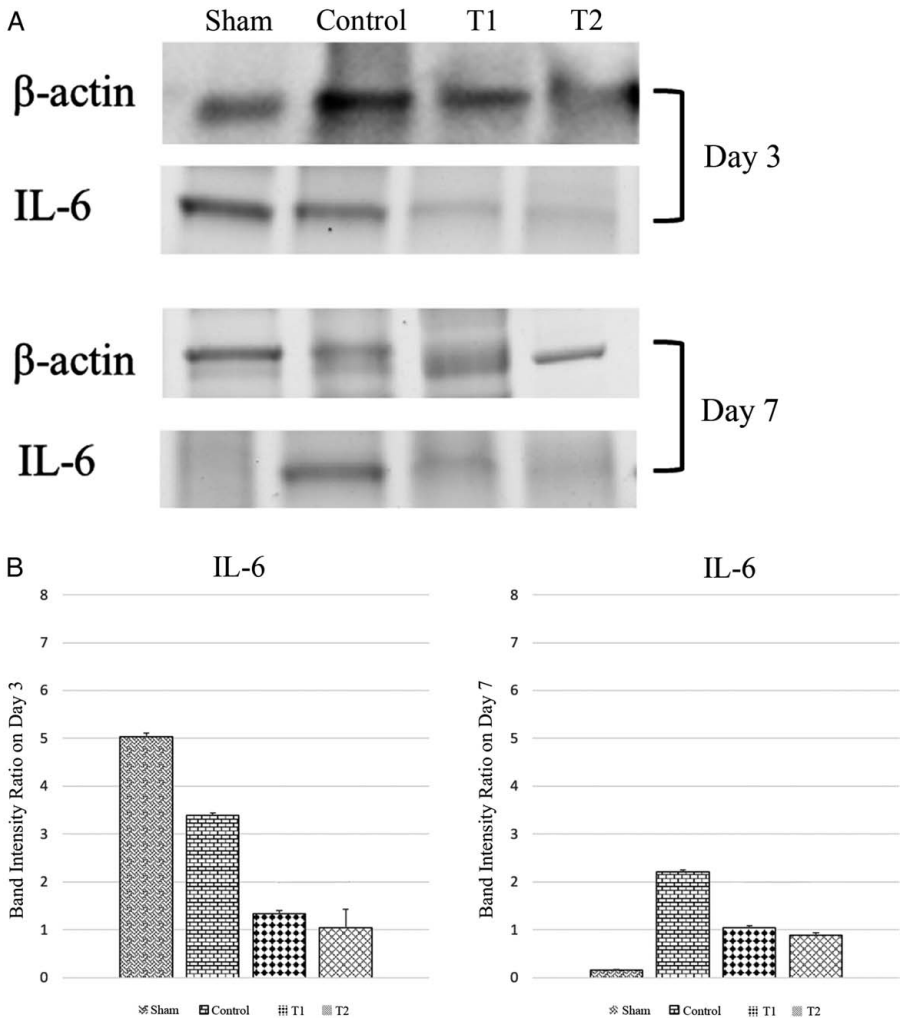

Figure 6. Interleukin-6 (IL-6) expression in duodenum of various study groups using western blot. (A) Thick band intensity showed an increase in IL-6 expression. There is also significant difference in IL-6 level between T1 and T2 groups ($P < 0.05$); (B) Quantification of IL-6 expression in duodenum of various study groups significant difference between control and treatment group.

MSCs wound healing potential

MSCs are critical players in the body's ability to heal and regenerate damaged tissues. These multipotent cells can differentiate into a variety of cell types, including osteoblasts, chondrocytes, myocytes, and adipocytes, making them invaluable in the repair of various tissue types. MSCs can be sourced from several tissues, such as bone marrow, adipose tissue, umbilical cord blood, and dental pulp. This versatility not only highlights their potential in regenerative medicine but also underscores their role in promoting healing across different tissues and organs. The basic biology of MSCs underpins their significance in medicine, particularly in enhancing the body's natural healing processes[12].

The ways in which MSCs contribute to wound healing are complex and multifaceted, involving various cellular and molecular processes. These stem cells facilitate the healing process by promoting cell migration, angiogenesis, epithelialization, and the formation of granulation tissue[13]. Additionally, MSCs have the ability to move into injured tissue areas and aid in repair by influencing the local environment to support healing[14]. They achieve this by releasing bioactive factors that enhance tissue repair and regeneration, such as the growth factor VEGF which is vital for skin wound healing[15].

In the realm of tissue regeneration, MSCs demonstrate impressive capabilities that extend beyond mere wound closure, contributing to the restoration of tissue function and structure. Their immunomodulatory, trophic, antibacterial, antifibrotic, and proangiogenic properties play a vital role in the early stages of wound healing by controlling and resolving inflammation to facilitate proper tissue repair and regeneration[14,16]. By modulating the inflammatory response and creating an optimal environment for tissue regeneration, MSCs are essential in ensuring that the healing processes result in functional tissue restoration rather than scar formation. These functions of MSCs highlight their potential as a valuable therapeutic tool for expediting the healing process and enhancing outcomes for patients with various types of wounds.

Several studies reported that umbilical cord MSCs can improve mucosal tissue regeneration in the inflammation process through numerous mechanisms. Hu *et al*.[17] revealed that MSCs can modulate macrophage response by polarizing it from pro-inflammatory M1 phenotype to anti-inflammatory M2 phenotype. This switch is a key step for mucosal wound healing and inflammation control. Additionally, MSCs also suppress pro-inflammatory TNF-a released by M1 and upregulate IL-10, one of the most potent anti-inflammatory cytokines secreted in acute phase[18].

IL-6 has pleiotropic properties as it has the ability to both enhance and suppress inflammation[19]. It is secreted by M1 macrophages to promote Th2 and Th17 differentiation into CD4 + T cells and inhibits proliferation by decreasing TGF-β-dependent differentiation of T-regulatory cells[20]. In the early phase of wound healing, its expression is increased, and it dramatically decreases as soon as the proliferation phase begins. As previously discussed above, inflammation has a role in lymphocyte infiltration into the wound site, which in turn secrete cytokines and growth factors[21]. Prolonged inflammation may result in an increased risk of wound dehiscence and scarring, which in turn may lead to reperforation of intestine sutures[1]. In this study, IL-6 expressions were significantly lower in T1 and T2 groups compared to the control group and further decreased on day 7. It demonstrates MSCs' role in reducing inflammation in the early phase of wound healing. These findings were observed directly in wound tissue, which justifies its role in duodenal tissues and is consistent with previous studies that report MSCs' role in reducing inflammation in various cases such as liver injury, lung infection, and gastric perforation[22–25]. According to these results, we propose the optimum dose of MSC for the treatment of duodenal perforation is 1.5×10^6 cells/10 mm.

Limitations and further recommendation

The limitations of this study include the limited sample size and the lack of adverse effects of MSCs administration is investigated. Thus, additional studies with bigger sample sizes and more thorough follow-ups are required to evaluate the possible negative effects of locally administered MSCs.

Conclusion

MSCs administration in rats reduces duodenal perforation inflammation by reducing leukocyte infiltration and IL-6 expression. Additional studies with bigger sample sizes and more thorough follow-ups are advised for future research to evaluate the possible negative effects of locally administered MSCs.

Ethical approval

Ethical approval has been given from the Commission of Medical Research Bioethics, Faculty of Medicine, Sultan Agung Islamic University with reference number: No.150/V/2021/Komisi Bioetik.

Consent

The present study followed international, national and/or institutional guidelines for humane animal treatment and complied with relevant legislation. Written informed consent are not required for this study.

Source of funding

Not applicable.

Author contribution

E.S. conceptualized the study and collected data. A.P. designed a laboratorium study and analyzed data. E.S., D.I.N., S.Z.O. and R.R. write the manuscript. All authors read and approved the final manuscript.

Conflicts of interest disclosure

The authors declare no conflict of interest.

Research registration unique identifying number (UIN)

Not applicable.

Guarantor

Eko Setiawan.

Data availability statement

The clinical and imaging data supporting the analysis and findings of this study will be available from the corresponding author upon reasonable request.

Provenance and peer review

It was not commissioned, externally peer-reviewed.

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