

Original Article

Hypoxia-conditioned mesenchymal stem cell secretome for anal sphincter injury: A preclinical study of tissue repair and regenerative outcomes

Leecarlo ML. Gaol^{1,2*}, Bryan A. Wijaya³, Yohanes Firmansyah³, Fiona V. Setiawan³, and Yuliana SK. Prabaningrum¹

¹Division of Pediatric Surgery, Department of Surgery, Tarakan General Hospital, Jakarta, Indonesia; ²Faculty of Medicine, Universitas Kristen Krida Wacana, Jakarta, Indonesia; ³Faculty of Medicine, Universitas Tarumanagara, Jakarta, Indonesia

*Corresponding author: millanoleecarlo@gmail.com

Abstract

Fecal incontinence may arise following anal sphincter injury, yet currently available treatments have limited capacity to restore sphincter integrity. The secretome derived from hypoxia-conditioned mesenchymal stem cells (MSCs) represents a potential cell-free regenerative therapy because of its putative immunomodulatory and tissue-remodeling properties. The aim of this study was to investigate the effects of hypoxia-conditioned human umbilical cord MSC secretome on inflammation, fibrosis, collagen deposition, and hypoxia-inducible factor-1 alpha gene (*HIF-1A*) expression following sphincterotomy. Male Sprague–Dawley rats were randomly assigned to receive either hypoxia-conditioned MSC secretome or 0.9% NaCl after surgical sphincterotomy. Histopathological evaluations using hematoxylin and eosin and Masson's trichrome staining were conducted at 24 hours and 42 days, and *HIF-1A* was assessed using quantitative real-time polymerase chain reaction (qRT-PCR). At day 42, inflammation scores were numerically lower in the secretome group than in the control group, although the between-group difference was not statistically significant (0.00 ± 0.00 vs 0.25 ± 0.25 ; $p=0.317$). Fibrosis scores showed a similar numerical pattern without a significant difference (3.25 ± 0.25 vs 3.50 ± 0.29 ; $p=0.495$). Conversely, the secretome group exhibited numerically greater collagen area ($79.93 \pm 11.11 \times 10^4 \mu\text{m}^2$ vs $59.25 \pm 7.44 \times 10^4 \mu\text{m}^2$; $p=0.149$) and percentage collagen area ($68.81 \pm 4.48\%$ vs $56.07 \pm 3.29\%$; $p=0.083$), but neither difference reached statistical significance. *HIF-1A* expression was also numerically higher in the secretome group at day 42, without a statistically significant between-group difference (27.47 ± 0.38 vs 26.44 ± 0.54 ; $p=0.149$). Although the secretome group exhibited numerically lower inflammation and fibrosis scores and greater collagen deposition and *HIF-1A* expression, these differences were not statistically significant. Further adequately powered studies incorporating functional, histological, and molecular endpoints are required to clarify the regenerative potential of hypoxia-conditioned MSC secretome in anal sphincter injury.

Keywords: Sphincterotomy, mesenchymal stem cell, secretome, inflammation, gene expression

Introduction

Fecal incontinence may result from structural or functional impairment of the anal canal and sphincter complex. Injury to the dentate line can impair anorectal sensation and the ability to



discriminate stool consistency and rectal distension, whereas damage to the internal or external anal sphincter can compromise continence mechanisms [1-4]. Such injuries may occur following iatrogenic resection, an excessively distal pull-through procedure, overstretching of the sphincter complex, myomectomy, or sphincterotomy [1-4]. Consequently, postoperative fecal incontinence may arise from the combined effects of impaired anorectal sensation, inadequate sphincter control, and abnormal colonic motility, resulting in persistent soiling and impaired continence [1-4].

Fecal incontinence is an important complication in children with Hirschsprung disease and anorectal malformations, particularly following reconstructive surgery [4-9]. Reported rates vary considerably across populations and clinical settings, ranging from approximately 22% to 73% in studies from Europe and North America [5-8]. Lower rates, ranging from approximately 2% to 7.8%, have generally been reported in Asian populations [4,9]. In children with anorectal malformations, fecal incontinence may also occur following posterior sagittal anorectoplasty (PSARP), emphasizing the importance of preserving and restoring sphincter integrity after surgical injury [4].

Current management strategies for fecal incontinence include enemas, neuromodulation, biofeedback, and surgical revision [10-12]. However, these approaches do not consistently restore sphincter integrity or achieve satisfactory continence, creating a need for therapeutic strategies that directly promote tissue repair. Mesenchymal stem cell (MSC)-derived secretome has emerged as a potential cell-free regenerative approach because it contains soluble bioactive mediators capable of regulating inflammation and promoting tissue repair [13]. The paracrine components of MSC secretome may modulate inflammatory responses and contribute to wound healing and extracellular matrix remodeling [13-16]. These components include transforming growth factor-beta 1 (TGF- β 1), interleukin (IL)-10, IL-18, IL-12-binding protein, IL-12p70, IL-17E, IL-27, ciliary neurotrophic factor, neurotrophin-3, and IL-1 receptor antagonist, which participate in the regulation of inflammatory and regenerative pathways [14-16].

The biological activity of MSC secretome may be further influenced by the conditions under which MSCs are cultured. Hypoxic preconditioning has been proposed as a strategy to enhance the regenerative properties of MSC secretome by inducing adaptive cellular responses and increasing the release of bioactive mediators involved in angiogenesis, fibroblast activity, and wound healing [20-23]. In particular, hypoxia-induced stabilization of hypoxia-inducible factor-1 alpha (HIF-1 α) may enhance signaling pathways involving vascular endothelial growth factor (VEGF), TGF- β 1, basic fibroblast growth factor (bFGF), and other mediators involved in extracellular matrix remodeling and tissue repair [20-23]. Despite these potential regenerative effects, the influence of hypoxia-conditioned MSC secretome on inflammation, fibrosis, and collagen remodeling following anal sphincter injury remains poorly characterized. Therefore, the aim of this study was to investigate the effects of hypoxia-conditioned MSC secretome on inflammation, fibrosis, collagen deposition, and HIF-1 α gene (*HIF-1A*) expression in a rat model of sphincterotomy-induced fecal incontinence.

Methods

Study design and experimental animals

This experimental study included 20 male Sprague-Dawley rats aged 12–16 weeks. Animals were obtained from a single breeding colony and randomly allocated in a 1:1 ratio to either the hypoxia-conditioned MSC secretome group or the 0.9% NaCl control group (n=10 per group). Animals were considered eligible when they were clinically healthy, with normal behavior and activity. Exclusion criteria included clinical evidence of illness, such as lethargy, poor coat condition, diarrhea, or bite wounds; a reduction in body weight of >10% during acclimatization; or death before initiation of the intervention. Before experimental procedures, all animals underwent a two-week acclimatization period under controlled laboratory conditions at Bogor Agricultural Institute, West Java, Indonesia.

Establishment of the sphincterotomy model

Before surgery, anesthesia was administered using intramuscular ketamine in combination with intraperitoneal xylazine hydrochloride at a dose of 0.15 mL/kg. Under microscopic guidance, a standardized anal sphincter injury involving approximately 25% of the sphincter complex was created. The internal and external anal sphincter muscles were separated, followed by standardized incisions at the 10 and 2 o'clock positions to achieve a consistent degree of sphincter injury across experimental animals.

Postoperative care and experimental endpoints

Following sphincterotomy, animals were monitored daily for clinical signs of pain and distress. Postoperative analgesia consisted of subcutaneous tramadol at 5 mg/kg body weight once daily for a minimum of three days. Oral paracetamol at 10 mg/kg body weight was administered twice daily when additional analgesia was required.

Experimental assessments were performed at 24 hours and 42 days after treatment. At the designated endpoints, animals were deeply anesthetized and euthanized by intracardiac administration of sodium pentobarbital at 200 mg/kg body weight in accordance with the approved animal welfare protocol. Following euthanasia, the abdomen was opened through a midline incision, and the distal large intestine extending to the anus was carefully dissected and harvested. The collected tissue was subsequently divided into two portions: one portion was fixed in paraformaldehyde for histological evaluation using hematoxylin–eosin and Masson's trichrome staining, whereas the remaining portion was immediately frozen and stored for subsequent analysis of *HIF-1A* gene expression.

Preparation of hypoxia-conditioned hUCMSC secretome

The secretome was prepared from human umbilical cord-derived MSCs (hUCMSCs). Umbilical cord tissue was collected and transported under sterile, cold conditions and subsequently washed to remove residual contaminants. The tissue was enzymatically digested using collagenase, after which the resulting cell suspension was sequentially filtered and centrifuged. Isolated cells were expanded in culture flasks with regular replacement of the culture medium.

Cells were maintained in T175 culture flasks for a maximum of six passages. Once 70–80% confluence was reached, the culture medium was replaced with baseline medium, and the cells were maintained under hypoxic conditions at 5% O₂ and 37°C for 72 hours. The resulting conditioned medium was collected and stored at –80°C until use. Characterization of the hypoxia-conditioned secretome showed pro-collagen and bFGF concentrations of 655,100.00 pg/mL and 34.64 pg/mL, respectively.

Administration of hypoxia-conditioned secretome

Immediately after establishment of the sphincterotomy model, animals assigned to the treatment group received a single 0.3-mL dose of hypoxia-conditioned MSC secretome by local intramuscular injection into the injured anal sphincter. The total volume was divided equally among three injection sites located at the 12, 4, and 8 o'clock positions, with 0.1 mL administered at each site. Animals assigned to the control group received 0.9% NaCl.

Histological assessments (inflammation and fibrosis)

Anal sphincter tissue specimens were fixed in 10% neutral-buffered formalin for 24 hours, subjected to routine tissue processing, embedded in paraffin, and sectioned using a microtome. Tissue sections were stained with hematoxylin and eosin (H&E) for evaluation of inflammatory cell infiltration and tissue architecture. Masson's trichrome staining was used to assess fibrosis and collagen deposition.

Inflammation and fibrosis were evaluated according to the Textor criteria [19]. Tissue involvement of <25% was assigned a score of 1, involvement of 25–50% a score of 2, involvement of 50–75% a score of 3, and involvement of >75% a score of 4. Four representative microscopic fields were evaluated for each specimen, and the mean score across these fields was used for statistical analysis.

Quantification of collagen deposition

Collagen deposition in Masson's trichrome-stained sections was quantitatively assessed using ImageJ (National Institutes of Health, Bethesda, USA). Two quantitative outcomes were evaluated: absolute collagen area, expressed in μm^2 , and the percentage of the evaluated tissue area occupied by collagen.

Quantitative real-time polymerase chain reaction

HIF-1A mRNA expression was assessed using quantitative real-time polymerase chain reaction (qRT-PCR). Messenger RNA was isolated from tissue samples using the Direct-zol™ RNA Kit (Zymo Research, Irvine, CA, USA), and RNA integrity was evaluated by electrophoresis. Complementary DNA (cDNA) was subsequently synthesized using the cDNA Synthesis System (Bioline, London, UK), according to the manufacturer's instructions. qRT-PCR was performed using SsoFast™ EvaGreen™ Supermix (Bio-Rad, Hercules, CA, USA) on a CFX Opus Real-Time PCR System (Bio-Rad, Hercules, CA, USA). β -actin gene (*ACTB*) was used as the internal reference gene. The amplification protocol consisted of an initial preincubation at 50°C for 2 minutes and enzyme activation at 95°C for 10 minutes, followed by 40 amplification cycles comprising denaturation at 94°C for 10 seconds, annealing at 55°C for 20 seconds, and extension at 72°C for 20 seconds. Relative gene expression was determined using the comparative threshold cycle (Ct) method. The primer sequences were as follows: *HIF-1A* forward, 5'-TGCTCATCAGTTGCCACTTC-3' and reverse, 5'-TGGGCCATTCTGTGTGTAA-3' [17]; and *ACTB* forward, 5'-CCTGTGCTGCTCACCGAGGC-3' and reverse, 5'-GACCCCGTCTCTCCGGAGTCC-ATC-3' [18].

Statistical analysis

Continuous variables were summarized as mean $\pm$ standard error (SE) together with 95% confidence intervals (CIs). Between-group differences in inflammation score, fibrosis score, *HIF-1A* expression, collagen area, and percentage collagen area were evaluated separately at 24 hours and 42 days using the Mann–Whitney U test. Statistical analyses were conducted using the Statistical Package for the Social Sciences (SPSS) (IBM, New York, USA).

Results

Experimental flow and final study population

Twenty rats entered the experiment following the two-week acclimatization period and were initially allocated equally to the hypoxia-conditioned mesenchymal stem cell (MSC) secretome and 0.9% NaCl control groups. Two animals died during the experimental period, including one during surgery and one on the first postoperative day. Consequently, 18 animals completed the study, with nine animals remaining in each group at day 42.

Inflammatory response following sphincterotomy

Inflammation scores decreased over the observation period in both groups (**Table 1; Figure 1**). At 24 hours, the mean inflammation score was numerically lower in the hypoxia-conditioned MSC secretome group than in the control group (2.40 ± 0.24 vs 2.50 ± 0.24), although the between-group difference was not statistically significant ($p=0.549$). At day 42, no detectable inflammation was reported in the secretome-treated group whereas the control group had a mean inflammation score of 0.25 ± 0.25 ($p=0.317$).

Table 1. Comparison of histological inflammation scores at 24 hours and 42 days after sphincterotomy between rats treated with 0.9% NaCl and hypoxia-conditioned mesenchymal stem cell (MSC) secretome

Time	Study group		p-value
	Saline control group	Hypoxia-conditioned MSC secretome group	
	Mean $\pm$ SE (95%CI)	Mean $\pm$ SE (95%CI)	
24 hours post-treatment	2.50 ± 0.24 (1.91–3.28)	2.40 ± 0.24 (1.72–3.08)	0.549
42 days post-treatment	0.25 ± 0.25 (-0.55–1.05)	0.00 ± 0.00	0.317

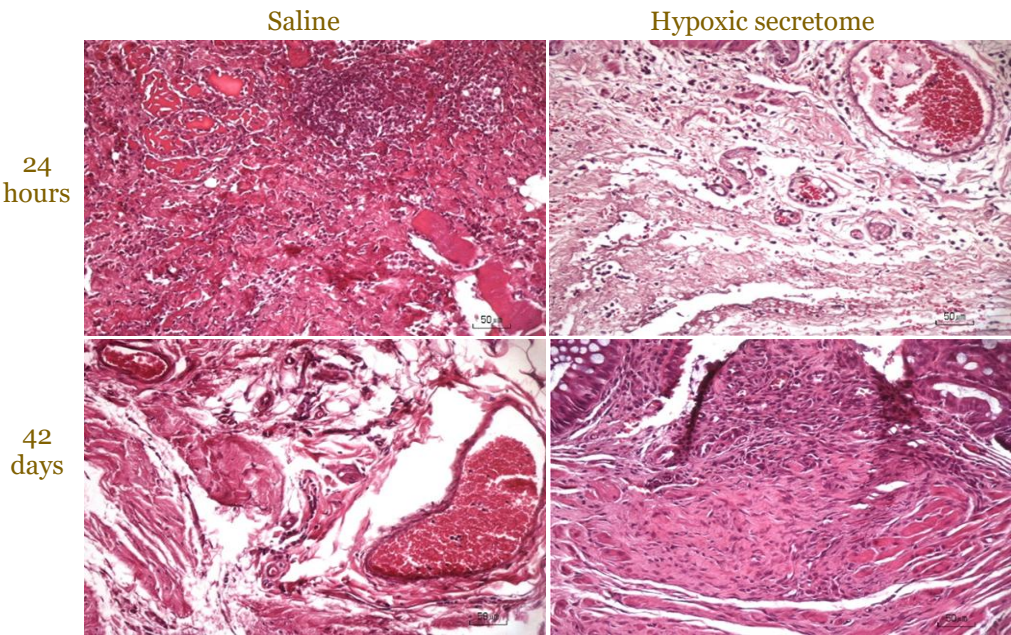


Figure 1. Inflammatory changes in anal sphincter tissue following sphincterotomy and treatment with hypoxia-conditioned mesenchymal stem cell (MSC) secretome.

Fibrotic response following sphincterotomy

Fibrosis scores increased between 24 hours and day 42 in both experimental groups (**Table 2; Figure 2**). At 24 hours, the mean fibrosis score was 1.60 ± 0.24 in the hypoxia-conditioned MSC secretome group and 1.40 ± 0.24 in the control group ($p=0.549$). At day 42, the mean fibrosis score was numerically lower in the secretome-treated group than in the control group (3.25 ± 0.25 vs 3.50 ± 0.29), although this difference did not reach statistical significance ($p=0.495$).

Table 2. Comparison of fibrosis scores at 24 hours and 42 days after sphincterotomy between rats treated with 0.9% NaCl and hypoxia-conditioned mesenchymal stem cell (MSC) secretome

Time	Study group		p-value
	Saline control group	Hypoxia-conditioned MSC secretome group	
	Mean±SE (95%CI)	Mean±SE (95%CI)	
24 hours post-treatment	1.40 ± 0.24 (0.72–2.08)	1.60 ± 0.24 (0.92–2.28)	0.549
42 days post-treatment	3.50 ± 0.29 (2.58–4.42)	3.25 ± 0.25 (2.45–4.05)	0.495

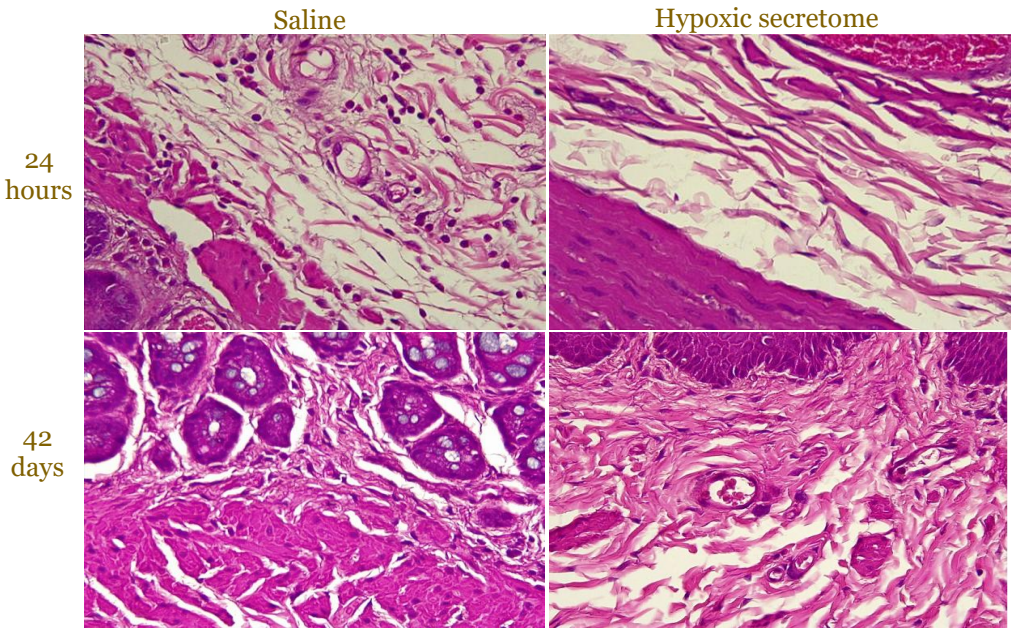


Figure 2. Fibrotic changes in anal sphincter tissue following sphincterotomy and treatment with hypoxia-conditioned mesenchymal stem cell (MSC) secretome.

Collagen deposition

Quantitative assessment of Masson's trichrome-stained sections showed temporal changes in collagen deposition in both groups (**Table 3; Figure 3**). At 24 hours, the mean collagen area was $38.03 \pm 5.65 \times 10^4 \mu\text{m}^2$ in the hypoxia-conditioned MSC secretome group and $40.58 \pm 6.58 \times 10^4 \mu\text{m}^2$ in the control group ($p=0.917$).

At day 42, the mean collagen area was numerically greater in the secretome-treated group than in the control group ($79.93 \pm 11.11 \times 10^4 \mu\text{m}^2$ vs $59.25 \pm 7.44 \times 10^4 \mu\text{m}^2$); however, the between-group difference was not statistically significant ($p=0.149$).

A similar temporal pattern was observed for the percentage of tissue area occupied by collagen (**Table 3**). At 24 hours, the percentage collagen area was $41.04 \pm 5.52\%$ in the secretome-treated group and $45.06 \pm 3.79\%$ in the control group ($p=0.465$). At day 42, the percentage collagen area was numerically greater in the hypoxia-conditioned MSC secretome group than in the control group ($68.81 \pm 4.48\%$ vs $56.07 \pm 3.29\%$), although the difference remained below the threshold for statistical significance ($p=0.083$) (**Table 3**).

Table 3. Comparison of collagen expression at 24 hours and 42 days after sphincterotomy between rats treated with 0.9% NaCl and hypoxia-conditioned mesenchymal stem cell (MSC) secretome

Indicator	Time	Study group		p-value
		Saline control group	Hypoxia-conditioned MSC secretome group	
		Mean $\pm$ SE (95%CI)	Mean $\pm$ SE (95%CI)	
Collagen area ($\times 10^4 \mu\text{m}^2$)	24 hours	40.58 $\pm$ 6.58 (22.32–58.84)	38.03 $\pm$ 5.65 (22.33–53.72)	0.917
	42 days	59.25 $\pm$ 7.44 (35.58–82.92)	79.93 $\pm$ 11.11 (44.54–115.31)	0.149
Collagen area (%)	24 hours	45.06 $\pm$ 3.79 (34.55–55.57)	41.04 $\pm$ 5.52 (25.71–56.36)	0.465
	42 days	56.07 $\pm$ 3.29 (45.60–66.54)	68.81 $\pm$ 4.48 (54.54–83.08)	0.083

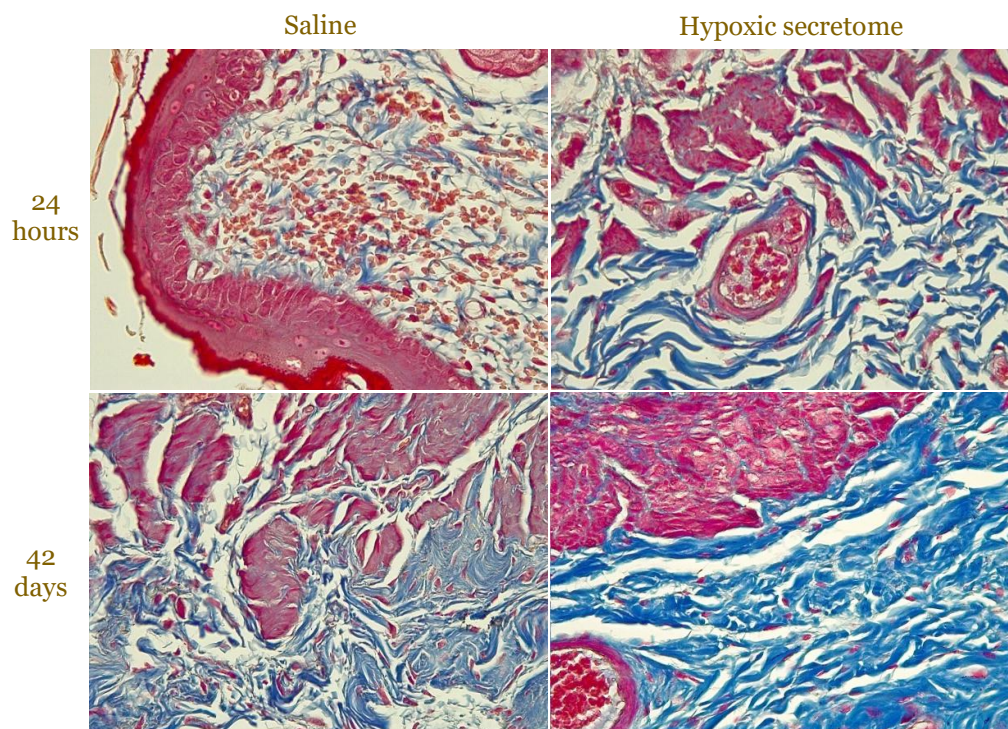


Figure 3. Collagen deposition in anal sphincter tissue following sphincterotomy and treatment with hypoxia-conditioned mesenchymal stem cell (MSC) secretome.

HIF-1A qRT-PCR findings

The reported *HIF-1A* qRT-PCR values were numerically higher in the hypoxia-conditioned MSC secretome group at both assessment points (**Table 4**). At 24 hours, the mean value was

24.97±0.23 in the secretome-treated group compared with 24.69±0.12 in the control group, with no statistically significant between-group difference ($p=0.251$). At day 42, the corresponding values were 27.47±0.38 and 26.44±0.54, respectively, and the difference remained statistically non-significant ($p=0.149$).

Table 4. Comparison of *HIF-1A* expression at 24 hours and 42 days after sphincterotomy between rats treated with 0.9% NaCl and hypoxia-conditioned mesenchymal stem cell (MSC) secretome

Time	Study group		p-value
	Saline control group	Hypoxia-conditioned MSC secretome group	
	Mean±SE (95%CI)	Mean±SE (95%CI)	
24 hours post-treatment	24.69±0.12 (24.37–25.01)	24.97±0.23 (24.32–25.61)	0.251
42 days post-treatment	26.44±0.54 (24.72–28.14)	27.47±0.38 (26.25–28.70)	0.149

Discussion

The principal finding of this study was that administration of hypoxia-conditioned MSC secretome following sphincterotomy was associated with numerical differences in several indices of tissue healing, although none of the between-group comparisons reached statistical significance. Compared with saline-treated controls, the secretome-treated group showed a numerically lower inflammatory score at 24 hours and no detectable inflammation at day 42, together with a slightly lower fibrosis score and greater collagen deposition at the later assessment. *HIF-1A* qRT-PCR values were also numerically higher in the secretome-treated group at both time points. Collectively, these findings indicate a possible biological effect of hypoxia-conditioned MSC secretome on inflammatory resolution and extracellular matrix remodeling after sphincter injury; however, they should be interpreted as preliminary trends rather than evidence of therapeutic efficacy.

Hypoxic preconditioning has been investigated as a strategy to enhance the regenerative properties of MSC-derived secretome. Exposure of MSCs to reduced oxygen tension induces cellular adaptive responses that can modify the composition of the secretome and increase the release of mediators involved in angiogenesis, tissue repair, and wound healing [20–23]. These responses are closely associated with HIF-1 α , a key regulator of cellular adaptation to hypoxia and downstream pathways involved in angiogenesis and tissue remodeling [20–23]. In the present study, the hypoxia-conditioned secretome contained pro-collagen at 655,100.00 pg/mL and bFGF at 34.64 pg/mL. The presence of these regenerative mediators provides biological plausibility for investigating hypoxia-conditioned secretome in sphincter repair, although the present study did not compare its composition directly with that of normoxia-conditioned secretome. Accordingly, the current findings cannot establish that hypoxic conditioning itself enhanced the secretome composition or therapeutic activity.

The observed inflammatory pattern may be related, at least in part, to interactions between hypoxic and inflammatory signaling pathways. Prolyl hydroxylase domain (PHD) proteins participate in oxygen-dependent regulation of HIF signaling, while alterations in PHD activity can also influence nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B)-mediated inflammatory responses [24,25]. Crosstalk between HIF-1 and NF- κ B may additionally involve competition for the transcriptional coactivator p300, providing a potential molecular link between cellular responses to hypoxia and inflammation [26–28]. Reciprocal regulation between these pathways has also been described through mechanisms involving inhibitor of nuclear factor kappa B alpha (I κ B α) and factor-inhibiting HIF [29,30]. Furthermore, PHD-related mechanisms have been implicated in the modulation of inflammatory signaling under hypoxic conditions [31–33]. In the present study, the numerically lower inflammation score at 24 hours and absence of detectable inflammation in the secretome-treated group at day 42 are directionally consistent with modulation of inflammatory responses. However, because the between-group differences were not statistically significant and inflammatory mediators were not directly measured, involvement of the HIF–NF- κ B pathway remains a mechanistic hypothesis rather than a demonstrated explanation for the observed histological findings.

The pattern of fibrosis and collagen deposition also requires careful interpretation. MSC-derived secretome contains diverse paracrine factors capable of influencing fibroblast activity, extracellular matrix turnover, inflammatory responses, and tissue repair [34-36]. Wound healing is a dynamic process involving overlapping inflammatory, proliferative, and remodeling phases, during which extracellular matrix deposition is followed by progressive structural reorganization [35]. In the present study, collagen area was initially similar between groups but was numerically greater in the hypoxia-conditioned secretome group at day 42, whereas the fibrosis score was slightly lower. This apparent divergence between collagen deposition and histological fibrosis may indicate differences in extracellular matrix organization or remodeling rather than simply greater fibrotic tissue accumulation. Nevertheless, collagen quantity alone cannot distinguish organized reparative matrix formation from pathological fibrosis, and the present study did not assess collagen subtype, fiber organization, matrix turnover, or biomechanical properties. The observed pattern should therefore not be interpreted as evidence that hypoxia-conditioned secretome reduced pathological fibrosis while simultaneously enhancing beneficial collagen formation.

Several molecular pathways could plausibly contribute to the observed extracellular matrix response. Hypoxia-related signaling can interact with phosphoinositide 3-kinase/protein kinase B (PI3K/Akt) pathways and influence HIF-1 α -dependent cellular responses [38,39]. Hypoxic conditioning of MSCs has also been shown to enhance their angiogenic paracrine activity, including increased VEGF production [40]. HIF-1 α signaling may subsequently interact with angiogenic and reparative pathways involving VEGF, while angiogenesis itself is closely integrated with tissue remodeling during wound repair [37,41]. Growth factors such as bFGF can also promote cellular proliferation and tissue repair during wound healing [42-45]. In addition, MSC-derived extracellular vesicles and secreted mediators have been reported to influence wound healing, matrix remodeling, and regenerative responses [46,47]. The greater collagen deposition observed at day 42 in the present study is biologically compatible with these mechanisms, but the study did not directly assess VEGF, TGF- β signaling, PI3K/Akt activation, angiogenesis, or extracellular matrix turnover. Therefore, any mechanistic link between hypoxia-conditioned secretome and the observed histological changes remains inferential.

Immunomodulatory effects of the MSC secretome may also contribute to tissue repair. MSC-derived soluble mediators have been reported to regulate immune-cell activity and alter the balance between pro-inflammatory and anti-inflammatory cytokine signaling [13-16]. Such effects may include modulation of tumor necrosis factor- α (TNF- α), interferon- γ (IFN- γ), interleukin (IL)-10, and other mediators involved in inflammatory resolution and tissue repair [13-16]. This immunoregulatory profile provides a plausible basis for the numerical reduction in inflammation observed in the present study. However, circulating or tissue concentrations of these cytokines were not measured, preventing direct attribution of the histological findings to secretome-mediated immunomodulation.

The absence of statistically significant between-group differences is an important consideration when interpreting the findings. Although 20 animals were initially included, two deaths resulted in nine animals per group completing the study. The relatively small final sample may have limited statistical precision, particularly for histological and molecular variables with substantial biological variability. Nevertheless, lack of statistical significance should not be attributed solely to sample size, nor should consistent numerical differences be regarded as evidence that a true treatment effect was present. The findings should instead be considered exploratory and hypothesis-generating. In addition, the study evaluated predominantly histological and molecular endpoints and did not assess anal sphincter contractility, continence, muscle regeneration, or other functional outcomes. The absence of a normoxia-conditioned MSC secretome group also prevents determination of whether hypoxic preconditioning provided additional biological benefit compared with conventional MSC secretome.

From a translational perspective, MSC-based regenerative strategies have previously been investigated for anal sphincter injury, with experimental studies reporting potential effects on muscle regeneration, tissue organization, and functional recovery [48,49]. Secretome-based therapy may offer conceptual advantages over direct cell transplantation because it delivers MSC-derived paracrine mediators without administration of viable cells [34,36,50]. Evidence from

preclinical wound-healing studies also supports further investigation of MSC-derived secretome and extracellular vesicles as cell-free regenerative approaches [50-55]. However, the present findings provide only preliminary histological and molecular evidence and are insufficient to support clinical application. Future studies should incorporate adequately powered experimental cohorts, normoxia-conditioned secretome comparators, standardized characterization of secretome components, dose-response assessments, protein-level validation of HIF-1 α and related signaling pathways, quantitative evaluation of extracellular matrix organization, and objective functional measures of sphincter recovery. Longer follow-up will also be required to determine whether early histological changes translate into sustained structural and functional improvement.

Some limitations should be considered when interpreting these findings. The relatively small sample size, further reduced by perioperative mortality, limited statistical precision and may have reduced the ability to detect modest between-group differences. The absence of a normoxia-conditioned MSC secretome comparator also prevents determination of whether the observed responses were specifically attributable to hypoxic preconditioning rather than to the secretome itself. The study primarily evaluated histological and molecular outcomes and did not include functional assessments of anal sphincter recovery, such as sphincter contractility or continence. In addition, *HIF-1A* was evaluated at the mRNA level without complementary protein-level validation, limiting mechanistic interpretation. Secretome characterization was restricted to pro-collagen and bFGF concentrations, while other potentially relevant cytokines, growth factors, extracellular vesicles, and bioactive mediators were not comprehensively assessed. Furthermore, collagen assessment was limited to total collagen area and percentage collagen area, which does not distinguish reparative extracellular matrix remodeling from pathological fibrosis. These limitations warrant confirmation in larger experimental studies incorporating appropriate comparator groups, comprehensive molecular characterization, and objective functional outcomes.

Conclusion

Hypoxia-conditioned MSC secretome showed numerical trends toward reduced inflammation and fibrosis and greater collagen deposition following sphincterotomy; however, none of the between-group differences reached statistical significance. These findings suggest a potential role of the secretome in modulating inflammatory responses and extracellular matrix remodeling during anal sphincter healing, but they do not establish therapeutic efficacy. Further studies with larger experimental cohorts, appropriate comparator groups, comprehensive molecular characterization, and functional assessment of sphincter recovery are required to confirm these preliminary findings and determine their translational relevance.

Ethics approval

The experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC), Faculty of Medicine, Padjadjaran University (approval no. 630/UN6.KEP/EC/2022). All experimental procedures were conducted in accordance with the approved animal welfare protocol. This study was conducted in accordance with ethical principles to ensure the welfare and humane treatment of the experimental animals.

Acknowledgments

None.

Competing interests

All the authors declare that there are no conflicts of interest.

Funding

This study received no external funding.

Underlying data

Derived data supporting the findings of this study are available from the corresponding author on request.

Declaration of artificial intelligence use

We hereby confirm that no artificial intelligence (AI) tools or methodologies were utilized at any stage of this study, including during data collection, analysis, visualization, or manuscript preparation. All work presented in this study was conducted manually by the authors without the assistance of AI-based tools or systems.

How to cite

Gaol LML, Wijaya BA, Firmansyah Y, *et al.* Hypoxia-conditioned mesenchymal stem cell secretome for anal sphincter injury: A preclinical study of tissue repair and regenerative outcomes. Narra J 2026; 6 (3): e3142 - <http://doi.org/10.52225/narra.v6i3.3142>.

References

1. Saheli M, Bayat M, Ganji R, *et al.* Human mesenchymal stem cells-conditioned medium improves diabetic wound healing mainly through modulating fibroblast behaviors. Arch Dermatol Res 2020;312(5):325-336.
2. Sevivas N, Teixeira FG, Portugal R, *et al.* Mesenchymal stem cell secretome: A potential tool for the prevention of muscle degenerative changes associated with chronic rotator cuff tears. Am J Sports Med 2017;45(1):179-188.
3. Van Koughnett JAM. Current management of fecal incontinence: Choosing amongst treatment options to optimize outcomes. World J Gastroenterol 2013;19(48):9216.
4. Putri GY, Wahid TBOR, Masdar H. Angka keberhasilan posterosagittal anorectoplasty (PSARP) yang dinilai dari skor Klotz pada pasien malformasi anorektal dibangsal bedah RSUD Arifin Achmad Provinsi Riau periode Januari 2009-Desember 2014. J Online Mhs Fak Kedokt Univ Riau 2014;1(2):1-8.
5. Minneci PC, Kabre RS, Mak GZ, *et al.* Can fecal continence be predicted in patients born with anorectal malformations? J Pediatr Surg 2019;54(6):1159-1163.
6. Bhargava P, Mahajan J, Kumar A. Anorectal malformations in children. J Indian Assoc Pediatr Surg 2006;11(3):136.
7. Peña A, Hong A. Advances in the management of anorectal malformations. Am J Surg 2000;180(5):370-376.
8. Langemeijer RATM, Molenaar JC. Continence after posterior sagittal anorectoplasty. J Pediatr Surg 1991;26(5):587-590.
9. Rajindrajith S, Devanarayana NM, Thapar N, *et al.* Functional fecal incontinence in children. J Pediatr Gastroenterol Nutr 2021;72(6):794-801.
10. Wald A. Diagnosis and management of fecal incontinence. Curr Gastroenterol Rep 2018;20(3):9.
11. Zhao X, Pasricha PJ. Novel surgical approaches to fecal incontinence: Neurostimulation and artificial anal sphincter. Curr Gastroenterol Rep 2003;5(5):419-424.
12. Tan JJY, Chan M, Tjandra JJ. Evolving therapy for fecal incontinence. Dis Colon Rectum 2007;50(11):1950-1967.
13. Ahangar P, Mills SJ, Cowin AJ. Mesenchymal stem cell secretome as an emerging cell-free alternative for improving wound repair. Int J Mol Sci 2020;21(19):7038.
14. Zagoura DS, Roubelakis MG, Bitsika V, *et al.* Therapeutic potential of a distinct population of human amniotic fluid mesenchymal stem cells and their secreted molecules in mice with acute hepatic failure. Gut 2012;61(6):894-906.
15. Li P, Ou Q, Shi S, *et al.* Immunomodulatory properties of mesenchymal stem cells/dental stem cells and their therapeutic applications. Cell Mol Immunol 2023;20(6):558-569.
16. Bermudez MA, Sendon-Lago J, Seoane S, *et al.* Anti-inflammatory effect of conditioned medium from human uterine cervical stem cells in uveitis. Exp Eye Res 2016;149:84-92.
17. Ueno M, Maeno T, Nomura M, *et al.* Hypoxia-inducible factor-1 α mediates TGF- β -induced PAI-1 production in alveolar macrophages in pulmonary fibrosis. Am J Physiol Lung Cell Mol Physiol 2011;300(5):L740-L752.
18. Arsenijevic T, Grégoire F, Delforge V, *et al.* Murine 3T3-L1 adipocyte cell differentiation model: Validated reference genes for qPCR gene expression analysis. PLoS One 2012;7(5):e37517.
19. Textor JA, Clark KC, Walker NJ, *et al.* Allogeneic stem cells alter gene expression and improve healing of distal limb wounds in horses. Stem Cells Transl Med 2018;7(1):98-108.
20. Dirja BT, Wardoyo EH, Putra A, *et al.* Enhanced third degree burn wound healing by hypoxic mesenchymal stem cells' secretome: IL-10 upregulation and TNF- α /PGE2 suppression. Trends Sci 2024;21(10):8288.

21. Jun E, Zhang Q, Yoon B, *et al.* Hypoxic conditioned medium from human amniotic fluid-derived mesenchymal stem cells accelerates skin wound healing through TGF- β /SMAD2 and PI3K/Akt pathways. *Int J Mol Sci* 2014;15(1):605-628.
22. Wang S, Umrath F, Cen W, *et al.* Pre-conditioning with IFN- γ and hypoxia enhances the angiogenic potential of iPSC-derived MSC secretome. *Cells* 2022;11(6):988.
23. Bartaula-Brevik S. Secretome of mesenchymal stem cells grown in hypoxia accelerates wound healing and vessel formation in vitro. *Int J Stem Cell Res Ther* 2017;4(1).
24. Ullah K, Rosendahl AH, Izzi V, *et al.* Hypoxia-inducible factor prolyl-4-hydroxylase-1 is a convergent point in the reciprocal negative regulation of NF- κ B and p53 signaling pathways. *Sci Rep* 2017;7(1):17220.
25. Han S, Xu W, Wang Z, *et al.* Crosstalk between the HIF-1 and Toll-like receptor/nuclear factor- κ B pathways in the oral squamous cell carcinoma microenvironment. *Oncotarget* 2016;7(25):37773-37789.
26. Mendonça DB, Mendonça G, Cooper LF. Mammalian two-hybrid assays for studies of interaction of p300 with transcription factors. *Methods Mol Biol* 2013;977:323-338.
27. Mendonça DBS, Mendonça G, Aragão FJL, *et al.* NF- κ B suppresses HIF-1 α response by competing for p300 binding. *Biochem Biophys Res Commun* 2011;404(4):997-1003.
28. Bandarra D, Biddlestone J, Mudie S, *et al.* HIF-1 α restricts NF- κ B-dependent gene expression to control innate immunity signals. *Dis Model Mech* 2015;8(2):169-181.
29. Shin DH, Li SH, Yang S, *et al.* Inhibitor of nuclear factor- κ B α derepresses hypoxia-inducible factor-1 during moderate hypoxia by sequestering factor inhibiting hypoxia-inducible factor from hypoxia-inducible factor 1 α . *FEBS J* 2009;276(13):3470-3480.
30. Müller-Edenborn K, Léger K, Glaus Garzon JF, *et al.* Hypoxia attenuates the proinflammatory response in colon cancer cells by regulating I κ B. *Oncotarget* 2015;6(24):20288-20301.
31. Stiehl DP, Wirthner R, Köditz J, *et al.* Increased prolyl 4-hydroxylase domain proteins compensate for decreased oxygen levels. *J Biol Chem* 2006;281(33):23482-23491.
32. Ginouvès A, Ilc K, Macías N, *et al.* PHDs overactivation during chronic hypoxia "desensitizes" HIF α and protects cells from necrosis. *Proc Natl Acad Sci U S A* 2008;105(12):4745-4750.
33. D'Angelo G, Duplan E, Boyer N, *et al.* Hypoxia up-regulates prolyl hydroxylase activity. *J Biol Chem* 2003;278(40):38183-38191.
34. Harrell C, Fellabaum C, Jovicic N, *et al.* Molecular mechanisms responsible for therapeutic potential of mesenchymal stem cell-derived secretome. *Cells* 2019;8(5):467.
35. Eming SA, Martin P, Tomic-Canic M. Wound repair and regeneration: Mechanisms, signaling, and translation. *Sci Transl Med* 2014;6(265):265sr6.
36. Vizoso F, Eiro N, Cid S, *et al.* Mesenchymal stem cell secretome: Toward cell-free therapeutic strategies in regenerative medicine. *Int J Mol Sci* 2017;18(9):1852.
37. Li M, Jia Q, Chen T, *et al.* The role of vascular endothelial growth factor and vascular endothelial growth inhibitor in clinical outcome of traumatic brain injury. *Clin Neurol Neurosurg* 2016;144:7-12.
38. Fojtík P, Beckerová D, Holomková K, *et al.* Both hypoxia-inducible factor 1 and MAPK signaling pathway attenuate PI3K/AKT via suppression of reactive oxygen species in human pluripotent stem cells. *Front Cell Dev Biol* 2021;8:607444.
39. Zhang Z, Yao L, Yang J, *et al.* PI3K/Akt and HIF-1 signaling pathway in hypoxia-ischemia: A review. *Mol Med Rep* 2018;18(4):3547-3554.
40. Hsiao ST, Lokmic Z, Peshavariya H, *et al.* Hypoxic conditioning enhances the angiogenic paracrine activity of human adipose-derived stem cells. *Stem Cells Dev* 2013;22(10):1614-1623.
41. Kretschmer M, Rüdiger D, Zahler S. Mechanical aspects of angiogenesis. *Cancers* 2021;13(19):4987.
42. Matsumoto S, Tanaka R, Okada K, *et al.* The effect of control-released basic fibroblast growth factor in wound healing. *Plast Reconstr Surg Glob Open* 2013;1(6):e44.
43. Pierce GF, Mustoe TA, Lingelbach J, *et al.* Platelet-derived growth factor and transforming growth factor- β enhance tissue repair activities by unique mechanisms. *J Cell Biol* 1989;109(1):429-440.
44. Zhang X, Kang X, Ji L, *et al.* Stimulation of wound healing using bioinspired hydrogels with basic fibroblast growth factor (bFGF). *Int J Nanomedicine* 2018;13:3897-3906.
45. Abdelhakim M, Lin X, Ogawa R. The Japanese experience with basic fibroblast growth factor in cutaneous wound management and scar prevention: A systematic review of clinical and biological aspects. *Dermatol Ther* 2020;10(4):569-587.
46. Gong X, Zhao Q, Zhang H, *et al.* The effects of mesenchymal stem cells-derived exosomes on metabolic reprogramming in scar formation and wound healing. *Int J Nanomedicine* 2024;19:9871-9887.

47. Liu C, Xu Y, Lu Y, *et al.* Mesenchymal stromal cells pretreated with proinflammatory cytokines enhance skin wound healing via IL-6-dependent M2 polarization. *Stem Cell Res Ther* 2022;13(1):414.
48. Mazzanti B, Lorenzi B, Borghini A, *et al.* Local injection of bone marrow progenitor cells for the treatment of anal sphincter injury: In-vitro expanded versus minimally-manipulated cells. *Stem Cell Res Ther* 2016;7(1):85.
49. Sarveazad A, Babahajian A, Yari A, *et al.* Combination of laser and human adipose-derived stem cells in repair of rabbit anal sphincter injury: A new therapeutic approach. *Stem Cell Res Ther* 2019;10(1):367.
50. Suhandi C, Mohammed AFA, Wilar G, *et al.* Effectiveness of mesenchymal stem cell secretome on wound healing: A systematic review and meta-analysis. *Tissue Eng Regen Med* 2023;20(7):1053-1062.
51. Wu D, Tao S, Zhu L, *et al.* Chitosan hydrogel dressing loaded with adipose mesenchymal stem cell-derived exosomes promotes skin full-thickness wound repair. *ACS Appl Bio Mater* 2024;7(2):1125-1134.
52. Zhu Y, Yang H, Xue Z, *et al.* Mesenchymal stem cells-derived small extracellular vesicles and apoptotic extracellular vesicles for wound healing and skin regeneration: A systematic review and meta-analysis of preclinical studies. *J Transl Med* 2025;23(1):364.
53. Tangsupati P, Murdiastuti K. The effect of collagen activation on platelet rich plasma for proliferation of periodontal ligament fibroblasts. *Indones Biomed J* 2018;10(3):278-283.
54. Sandra F, Sudiono J, Sidharta EA, *et al.* Conditioned media of human umbilical cord blood mesenchymal stem cell-derived secretome induced apoptosis and inhibited growth of HeLa cells. *Indones Biomed J* 2014;6(1):57.
55. Meiliana A, Dewi NM, Wijaya A. Mesenchymal stem cell secretome: Cell-free therapeutic strategy in regenerative medicine. *Indones Biomed J* 2019;11(2):113-124.