



Effects of Hypoxic Adipose-Derived Mesenchymal Stem Cells and Their Secretome on Type II Collagen Expression in a Rabbit Model of Disc Degeneration

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ABSTRACT

Introduction: Intervertebral disc (IVD) degeneration is a major cause of low back pain, and is characterized by the loss of extracellular matrix, particularly type II collagen. Regenerative therapies using mesenchymal stem cells (MSCs) and their secretome have shown promise, but their comparative effects on type II collagen expression require further investigation. This study aimed to evaluate and compare the effects of allogeneic hypoxic adipose-derived mesenchymal stem cells (Ad-MSCs) and their secretome on type II collagen expression in a rabbit model of IVD degeneration. **Methods:** A true experimental study was conducted on 18 New Zealand White rabbits. Degeneration was induced by needle puncture. Subjects were divided into three groups: control (fibrin glue), P1 (hypoxic Ad-MSC), and P2 (secretome). After 8 weeks, type II collagen expression was analyzed using immunohistochemistry. **Results:** Both P1 and P2 showed significantly higher type II collagen expression than the control ($p = 0.001$ and $p < 0.001$, respectively). The secretome group (P2) showed significantly higher expression than the Ad-MSC group (P1) ($p = 0.039$). These findings suggest that the secretome may provide a greater stimulatory effect on type II collagen expression than direct cell administration. **Conclusion:** The hypoxic Ad-MSC-derived secretome resulted in higher type II collagen expression than hypoxic Ad-MSCs administration in this rabbit model.

Keywords: Ad-MSCs, intervertebral disc degeneration, rabbit model, secretome, type II collagen.



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INTRODUCTION

Intervertebral disc (IVD) degeneration is a debilitating condition and a primary cause of low back pain, imposing a significant socioeconomic burden worldwide.¹ The degenerative process is fundamentally characterized by the progressive loss of the extracellular matrix (ECM) within the nucleus pulposus (NP).² In a healthy disc, the NP is a gelatinous structure rich in proteoglycans and type II collagen, which are essential for maintaining hydration and hydrostatic pressure.³ During degeneration, there is a distinct shift in matrix composition where the synthesis of type II collagen decreases significantly.⁴ This healthy collagen is often replaced by the more fibrous collagen type I.⁴ This phenotypic switch compromises the disc's biomechanical competence, leading to structural failure and pain.⁵

Current therapeutic strategies, including

pharmacological management and surgical interventions, are predominantly symptomatic or structural.⁶ These conventional treatments do not directly replace the biologically altered disc matrix.⁶ Consequently, regenerative medicine has emerged as a potential strategy to modify disc biology.⁷ Mesenchymal stem cells (MSCs), particularly adipose-derived MSCs (Ad-MSCs), have attracted interest because of their chondrogenic potential and secretion of bioactive factors.^{6,7} Increasing evidence suggests that some MSC effects are mediated through paracrine products collectively termed the secretome, which contains growth factors, cytokines, and extracellular vesicle-associated signals capable of modifying the local microenvironment.^{6,8,9}

Hypoxic preconditioning can increase MSC secretion of regenerative factors.⁸ However, the comparative effects of hypoxic

Ad-MSCs and their cell-free secretome on type II collagen expression in the disc remain incompletely defined. This study aimed to evaluate and compare the effects of allogeneic hypoxic Ad-MSCs and their secretome on type II collagen expression in a rabbit model of induced IVD degeneration. We hypothesized that both hypoxic Ad-MSCs and their secretome would increase type II collagen expression compared with the control, with the secretome producing a greater effect.

Indonesian clinical reports have documented low-back-pain-related quality-of-life impairment in older adults and occupational risk factors among bus drivers.^{10,11} Within the literature cited in this manuscript, we found no Indonesian preclinical study directly comparing hypoxic Ad-MSCs with their secretome for IVD degeneration. In an Asian rabbit model, Chiang, et al., compared

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allogeneic hypoxic and normoxic MSCs and assessed type II collagen deposition,¹² whereas Gui, *et al.*, used a 16G needle advanced 5 mm in New Zealand White rabbits and evaluated type II collagen by immunohistochemistry.¹³ The present study builds on these models by directly comparing allogeneic hypoxic Ad-MSCs with their cell-free secretome using type II collagen expression as the prespecified outcome.

MATERIALS AND METHODS

Study Design and Animal Model

This study employed a true experimental laboratory design with a randomized post-test-only control-group structure. The study was conducted from December 2020 to May 2023. The study used 18 skeletally mature male New Zealand White rabbits (4–5 months old, weighing 3.25–3.75 kg). The research protocol was approved by the Animal Care and Use Committee (ACUC), Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia, under ethical clearance No. 2.KE.098.11.2020, dated November 3, 2020. Subjects were randomly assigned to three groups ($n = 6$ per group) using a computer-generated random-number table.

Intervertebral disc degeneration was induced under general anesthesia (ketamine 40 mg/kg and xylazine 5 mg/kg). A posterolateral retroperitoneal approach was used to expose the lumbar spine. Degeneration was induced by controlled puncture of the L4–L5 disc with a 16G needle advanced 5 mm, consistent with a previously described rabbit model.¹³ Treatment was administered 4 weeks after puncture, the interval used for model establishment; a representative 4-week degeneration specimen is shown in **Figure 2M**. We defined model establishment by the standardized puncture procedure and 4-week induction interval. This report did not include subject-level pre-treatment imaging or biopsy data; therefore, degeneration could not be independently verified in each treated disc before intervention.

Treatment Groups and Interventions

Four weeks post-induction, the animals received the following percutaneous intradiscal injections:

- **Control Group (K1):** Received fibrin glue (0.1 mL).

- **Stem Cell Group (P1):** Received 2×10^6 allogeneic hypoxic Ad-MSCs suspended in fibrin glue. The available study record did not document the final injected volume.
- **Secretome Group (P2):** Received hypoxic Ad-MSC-derived secretome (conditioned medium; 100 $\mu\text{g/mL}$ total protein) mixed with fibrin glue. The final injected volume was not documented; therefore, the available record could not be used to calculate the absolute protein dose delivered per disc.

We isolated Ad-MSCs from rabbit adipose tissue and cultured them under hypoxic conditions (3% O_2) to enhance their paracrine profile. The secretome was harvested from the supernatant after a 48-hour conditioning period. The conditioned medium was centrifuged at $3,000 \times g$ for 10 minutes to remove cellular debris, filtered through a 0.22 μm PVDF membrane, concentrated 10-fold using Amicon Ultra-15 centrifugal filters (3-kDa molecular-weight cutoff), and normalized to 100 $\mu\text{g/mL}$ total protein before injection.

Immunohistochemical Analysis of Type II Collagen

The animals were terminated 8 weeks after treatment administration. The L4–L5 intervertebral discs were harvested, fixed in 10% buffered formalin, decalcified, and embedded in paraffin. Serial sections of 4 μm thickness were prepared for immunohistochemical (IHC) staining.

The tissue sections were deparaffinized, rehydrated, and subjected to antigen retrieval. They were then incubated with a primary monoclonal antibody against rabbit type II collagen (anti-collagen II). A secondary antibody conjugated with HRP was applied, followed by DAB chromogen visualization and hematoxylin counterstaining.

Expression of type II collagen was quantified by evaluating brown staining intensity in the nucleus pulposus. IHC assessment was performed using a light microscope at 400 $\times$ magnification by two independent pathologists who were blinded to the treatment groups. Expression was scored semiquantitatively using the modified

Remmele immunoreactive score (IRS), which combines the percentage of positive cells and staining intensity.¹⁴

Statistical Analysis

Data were expressed as mean $\pm$ standard deviation (SD). The original analysis assessed normality using the Shapiro–Wilk test and compared groups using one-way analysis of variance (ANOVA) followed by Tukey's HSD post-hoc test; a two-sided p value < 0.05 was considered statistically significant. The available analysis record did not include the exact group-specific Shapiro–Wilk statistics or the SPSS version. Individual-animal IRS values were also unavailable during revision, precluding a Kruskal–Wallis/Dunn reanalysis. As a supplementary robustness check, we reconstructed Welch's ANOVA and pairwise Welch t tests with Holm adjustment from the reported group sizes, means, and SDs.

RESULTS

Analysis of Type II Collagen Expression

To evaluate the interventions' effects on the prespecified outcome, type II collagen expression was quantified using immunohistochemical staining. Descriptive analysis showed differences in expression across the experimental groups at 8 weeks.

The control group (K1), which received fibrin glue, had the lowest mean IRS (1.651 ± 0.120). The hypoxic Ad-MSC group (P1) had a mean score of 2.073 ± 0.147 , and the secretome group (P2) had the highest mean score of 2.378 ± 0.230 (**Figures 1 and 2**). One-way ANOVA indicated a significant between-group difference ($p < 0.001$; **Table 1**).

Post-hoc analysis showed that both treatment groups had significantly higher type II collagen expression than the control group (P1 vs K1, $p = 0.001$; P2 vs K1, $p < 0.001$) (**Table 2**). Direct comparison showed that P2 had significantly higher type II collagen expression than P1 ($p = 0.039$). This result indicates a larger treatment-associated effect on the measured type II collagen outcome in the secretome group; it does not, by itself, demonstrate broader matrix regeneration or functional recovery.

A summary-data sensitivity analysis confirmed the overall between-group difference (Welch

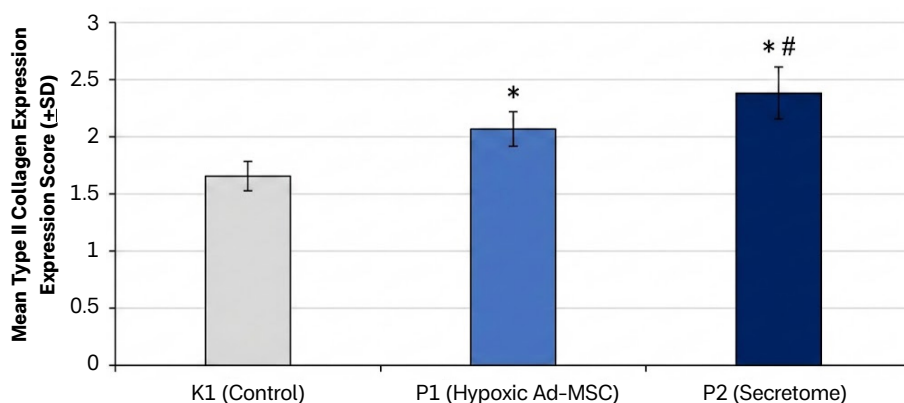


Figure 1. Quantitative analysis of type II collagen expression in the nucleus pulposus at 8 weeks post-treatment. The bar chart represents the mean immunoreactive score ($\pm$ standard deviation). (* indicates $p < 0.05$ compared to the K1 control group; # indicates $p < 0.05$ compared to the P1 hypoxic Ad-MSC group; one-way ANOVA followed by Tukey's HSD post-hoc test).

Table 1. Mean expression of type II collagen at 8 weeks post-treatment.

Group	Treatment	Amount (n)	Type II Collagen Expression (Mean Score $\pm$ SD)
K1	Control (Fibrin Glue)	6	1.651 $\pm$ 0.120
P1	Hypoxic Ad-MSC	6	2.073 $\pm$ 0.147
P2	Secretome	6	2.378 $\pm$ 0.230

Note: One-way ANOVA indicated a significant difference among groups ($p < 0.001$).

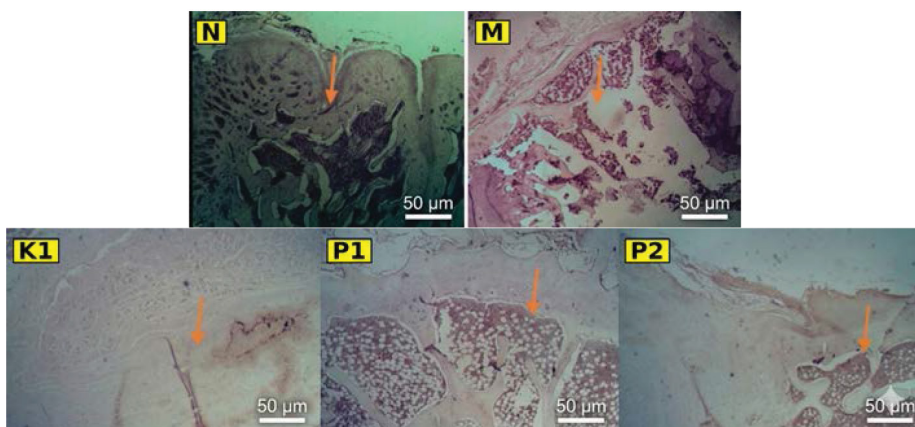


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Figure 2. Immunohistochemical staining of type II collagen. (N) Normal disc; (M) Degeneration model (4 weeks post-puncture); (K1) Control (fibrin glue); (P1) Ad-MSC treatment; (P2) Secretome treatment. All treatment groups were evaluated at 8 weeks. (Scale bar: 50 μ m; magnification: 400 $\times$). Panel order is N and M (top row, left to right) and K1, P1, and P2 (bottom row, left to right).

Table 2. Multiple comparisons (post-hoc test) of type II collagen expression.

Comparison	Mean Difference (SE)	95% CI	p-value	Interpretation
P1 vs K1	0.422 (0.106)	0.147 to 0.697	0.001*	P1 significantly higher than control
P2 vs K1	0.727 (0.106)	0.452 to 1.002	< 0.001*	P2 significantly higher than control
P2 vs P1	0.305 (0.106)	0.030 to 0.580	0.039*	P2 significantly higher than P1

Note: *The mean difference is significant at the 0.05 level. CI, confidence interval; SE, standard error. Simultaneous 95% CIs were reconstructed from the reported mean differences and SEs using Tukey's studentized-range critical value (three groups, $df = 15$); minor differences from the original software output may occur because the source values were rounded.

$F(2, 9.50) = 28.06$, $p < 0.001$). Holm-adjusted pairwise Welch tests remained significant for P1 versus K1 ($p < 0.001$), P2 versus K1 ($p < 0.001$), and P2 versus P1 ($p = 0.024$). Because these calculations were reconstructed from rounded summary statistics rather than individual-animal data, they should be interpreted as a robustness check and not as a substitute for a rank-based analysis.

DISCUSSION

The primary finding was that both allogeneic hypoxic Ad-MSCs and their secretome were associated with higher type II collagen expression than the fibrin glue control, and the secretome group had higher expression than the Ad-MSC group. Because type II collagen was the only matrix marker quantified, these data demonstrate a treatment-associated difference in this marker but do not establish global extracellular matrix restoration, cell engraftment, or functional disc regeneration.

Type II collagen is a major component of the healthy nucleus pulposus extracellular matrix and contributes to disc hydration and load distribution.^{2,3} Lower expression in K1 and higher expression in P1 and P2 indicate differences in this matrix marker. Catabolic mediators, cell phenotype, proteoglycan content, imaging, and biomechanics were not directly assessed; therefore, the findings should not be interpreted as proof that the overall catabolic process was reversed or that resident cells redifferentiated.

The greater type II collagen expression in P2 than P1 is consistent with, but does not prove, the paracrine hypothesis that MSC-derived bioactive products can mediate therapeutic effects.^{6,9} Secretome preparations contain soluble factors and extracellular vesicle-associated signals that may influence recipient cells.^{8,9} Because secretome



composition and cell engraftment were not measured, the mechanism underlying the observed difference remains inferential.

One possible explanation is that transplanted MSCs must remain viable in the acidic, nutrient-poor, inflammatory environment of a degenerated disc, whereas secretome administration delivers cell-derived products directly.^{6,15} However, this study did not measure cell survival, local cytokines, or uptake of secretome components.

Our study used 3% O₂ hypoxic preconditioning, which may have enhanced the paracrine profile of Ad-MSCs.⁸ Hypoxic preconditioning can alter MSC secretion through hypoxia-responsive pathways, but we did not quantify HIF-1 α , TGF- β , IGF-1, cytokines, or extracellular vesicles. Therefore, the present data cannot identify which secretome components mediated the observed difference.

Cell-free secretome approaches may offer practical advantages over live-cell therapy, including simpler storage and avoiding administration of viable cells.^{6,9} Nevertheless, safety, immunogenicity, dose-response, biodistribution, or functional efficacy were not evaluated, so these results should be regarded as preclinical evidence limited to type II collagen expression.

These findings can be compared with Chiang, *et al.*, who reported greater type II collagen deposition after hypoxic than normoxic allogeneic MSCs treatment in a rabbit puncture model.¹² The present study extends that comparison to an Ad-MSC-derived secretome; however, it did not include a normoxic comparator. Accordingly, 3% O₂ should not be described as the optimal culture condition, and the higher IRS in P2 cannot be attributed specifically to hypoxia without additional comparator groups and secretome profiling.

This study has several limitations. The 8-week follow-up was short, and the study did not assess structural, molecular, imaging, biomechanical, or functional outcomes. Aggrecan and other matrix markers were not quantified. An a priori power calculation was not reported, so the statistical adequacy of

$n = 6$ per group cannot be fully evaluated. Ad-MSC identity was not documented by surface-marker profiling or trilineage differentiation. Subject-level pre-intervention imaging or biopsy data were unavailable, so degeneration was not independently verified in each treated disc before therapy. The final injected volumes for P1 and P2 were not documented, preventing calculation of the absolute secretome protein dose. The exact Shapiro–Wilk output and individual-animal IRS values were unavailable, precluding a Kruskal–Wallis/Dunn reanalysis. However, the summary-data Welch sensitivity analysis supported the reported findings; the small sample and semiquantitative outcome require cautious interpretation. Finally, rabbit spinal loading differs from human bipedal loading, limiting direct translation.

CONCLUSION

In this rabbit model of intervertebral disc degeneration, intradiscal administration of allogeneic hypoxic Ad-MSCs and their secretome was associated with higher type II collagen expression than the fibrin glue control. The secretome group demonstrated significantly higher type II collagen expression than the Ad-MSC group. These findings suggest that the hypoxic Ad-MSC-derived secretome may be a cell-free approach to enhance extracellular matrix-associated type II collagen expression. Further studies assessing long-term structural, molecular, and functional outcomes are warranted.

DECLARATIONS

Ethics Statement

The research protocol was approved by the Animal Care and Use Committee (ACUC), Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia, under ethical clearance No. 2.KE.098.11.2020, dated November 3, 2020. All experimental procedures involving animals were conducted in accordance with the approved research protocol and applicable institutional guidelines for the ethical care and use of laboratory animals.

Consent for Publication

Not applicable. This study involved experimental animals and did not include human participants, identifiable personal information, or images of identifiable individuals. All authors have reviewed and approved the final manuscript and consented to its publication.

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Author Contributions

Romaniyanto: Conceptualization, study design, methodology, investigation, experimental procedures, data acquisition, resources, supervision, project administration, interpretation of results, and critical revision of the manuscript. Dykall Naf'an Dzikri: Data curation, formal analysis, interpretation of results, literature review, manuscript preparation, writing of the original draft, and critical revision of the manuscript. Daffa Sadewa: Data collection, literature review, manuscript preparation, and writing, review, and editing.

Muhammad Setya Fachreza: Data collection, literature review, manuscript preparation, and writing, review, and editing.

All authors reviewed and approved the final version of the manuscript and agreed to be accountable for all aspects of the work.

Conflict of Interest

The authors declare that they have no financial or non-financial conflicts of interest related to the conduct of this study or the publication of this manuscript.

Declaration on the Use of Artificial Intelligence (AI)

The authors declare that artificial intelligence (AI) assistance was utilized during the preparation of this manuscript. Specifically, OpenAI's ChatGPT was used to assist with language editing, grammatical correction, sentence restructuring, and manuscript drafting and revision to improve clarity, readability, and organization.

AI-generated content was critically reviewed and verified by the authors before inclusion in the manuscript. The authors retained full responsibility for the manuscript's scientific accuracy, integrity, originality, and final content. All authors reviewed, verified, and approved the final version of the manuscript.

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