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Effect of umbilical cord mesenchymal stem cell-derived mitochondrial transplantation on ischemia-reperfusion injury in a rat model

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Abstract

Background: Despite advancements in reconstructive procedures, ischemia-reperfusion (I/R) injury remains a significant challenge in reconstructive surgery, with mitochondrial dysfunction playing a pivotal role. Mitochondrial transplantation has emerged as a promising therapeutic strategy to address this issue. This study aims to evaluate the impact of umbilical cord mesenchymal stem cell-derived mitochondrial transplantation on skin flap I/R models in rats.

Material and methods: Twenty male rats underwent I/R injury on skin flaps, with or without mitochondrial transplantation administered via intravenous or subcutaneous routes. Analysis encompassed histopathology, inflammatory, apoptotic, oxidative stress, and hypoxia markers.

Results: Results revealed a reduction in inflammation, apoptosis, oxidative stress, and hypoxia in the transplantation group compared to controls.

Conclusion: The findings suggest that umbilical cord mesenchymal stem cell-derived mitochondrial transplantation shows promise in enhancing flap viability and attenuating I/R injury, offering valuable insights for improved outcomes in reconstructive surgery. However, further exploration in larger animal models and refinement of delivery methods and dosage are warranted to fully elucidate its clinical translatability.

KEYWORDS
ischemia reperfusion injury, mitochondria, umbilical cord mesenchymal stem cells

1 | INTRODUCTION

Reconstructive procedures in plastic and reconstructive surgery, such as replantation, free tissue transfer, composite tissue allotransplantation, and various reconstructive flap surgeries, are commonplace. Despite the prosperous outcomes, the challenge of ischemia-reperfusion (I/R) injury persists, primarily due to the sudden restoration of circulation following prolonged ischemia, leading to a significant risk of partial to total flap loss. Mitochondrial dysfunction has been identified as a pivotal factor in the intricate pathways of I/R injury.[1] Phosphocreatine produced by mitochondria is one of the factors which allow skin to survive ischemia associated with skin flaps. These enzymatic processes preceded by mitochondrial respiration which is known to be essential for skin functions as it produces the main source of ATP through the oxidative phosphorylation process.[2]

Addressing this challenge, mitochondrial transplantation has emerged as a promising therapeutic strategy. This technique involves delivering mitochondria to the target organ through direct injection or

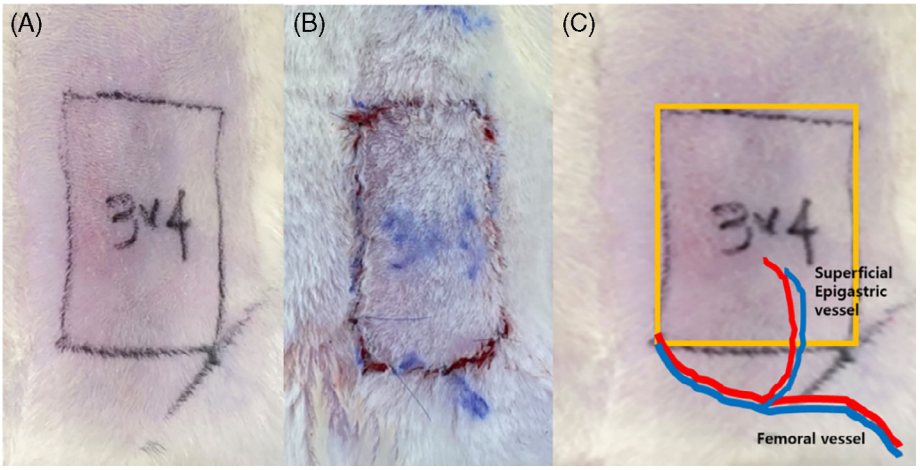

FIGURE 1 Left inferior epigastric flap as a rat I/R model. (A) Preoperative skin marking photograph, (B) Postoperative photograph, (C) Schematic illustration of the experimental model.

vascular infusion, offering a rapid and direct method.[3] Since its initial demonstration as a potential therapy in 2008,[4] numerous studies have developed methods to measure mitochondrial metabolism[5] and have highlighted its efficacy in mitigating I/R injury across various animal models of critical illness.[3,5,6]

This study aims to assess the effect of umbilical cord mesenchymal stem cell-derived mitochondrial transplantation on skin flaps in a rat I/R model. Furthermore, it seeks to compare the effectiveness of two distinct delivery methods of mesenchymal stem cell-derived mitochondria on skin flaps in a rat I/R model.

2 | MATERIAL AND METHODS

Twenty male Sprague–Dawley (SD) rats, aged 8 weeks and weighing between 250 and 300 g, were used for the surgical procedures following the Guidelines for Animal Experimentation of Soonchunhyang University School of Medicine, Korea (SCHBCA2022-07). Before the commencement of the study, a 7-day acclimation period was provided for the rats under standard conditions. They were housed with unrestricted access to water and standard rodent diet, maintaining a controlled environment at a temperature of 23°C ± 1°C, a relative humidity of 45%–55%, and subjected to a 12-h light/dark cycle (lights on from 08:00 to 20:00).

The procedure was performed after ketamine-xylazine intraperitoneal i/p injection (Ketamine, 90 mg/kg; Xylazine, 10 mg/kg). In all rats, a rectangular-shape flap measuring 4 × 3 cm, based by the left superficial inferior epigastric (SIE) vessels (Figure 1), was raised. This flap comprised the skin, underlying subcutaneous tissue, and closely associated panniculus carnosus.

2.1 | Study design

The rats were divided into two main groups: control and transplantation. Within the transplantation group, subgroups were established based on the method of mitochondrial delivery – either intravenous (I/V) administration before or after, or subcutaneous (S/C) administration before or after the induction of I/R (Figure 2).

Control group ($n = 4$): Rats in this group were subjected to I/R injury on the skin flap without any intervention. The procedure included inducing 3 h of flap ischemia by applying a 1 mm microvascular clip with 13.5 g compression on the pedicle. During this time, the rats remained anesthetized, and the flap was temporarily sealed with nylon. Upon removing the clip, the integrity of the blood vessels in the pedicles was verified using a microscope before repositioning and suturing the flap.

Transplantation group ($n = 16$): Rats in this group underwent umbilical cord mesenchymal stem cell-derived mitochondrial transplantation via intravenous or subcutaneous routes. All other surgical procedures were identical to those performed on the control group.

I/V before ($n = 4$): Rats in this subgroup received 100 μg of umbilical cord mesenchymal stem cell-derived mitochondria via intravenous injection into the tail vein 30 min prior to the flap ischemia.

I/V after ($n = 4$): Rats in this subgroup received 100 μg of umbilical cord mesenchymal stem cell-derived mitochondria via intravenous injection into the tail vein 30 min after the clamping of epigastric artery.

S/C before ($n = 4$): Rats in this subgroup received 100 μg of umbilical cord mesenchymal stem cell-derived mitochondria via subcutaneous injection, directed into the center of the flap, 30 min prior to the clamping of the epigastric artery.

S/C after ($n = 4$): Rats in this subgroup received 100 μg of umbilical cord mesenchymal stem cell-derived mitochondria via subcutaneous injection, directed into the center of the flap, 30 min after the clamping of the epigastric artery.

2.2 | Isolation of mitochondria from UC-MSC

UC-MSC were used at passage 7 for mitochondria preparation. Cells were harvested from culture flasks, depressurized in SHE buffer (0.25 M Sucrose, 20 mM HEPES [pH 7.4], 2 mM EGTA, 0.1%

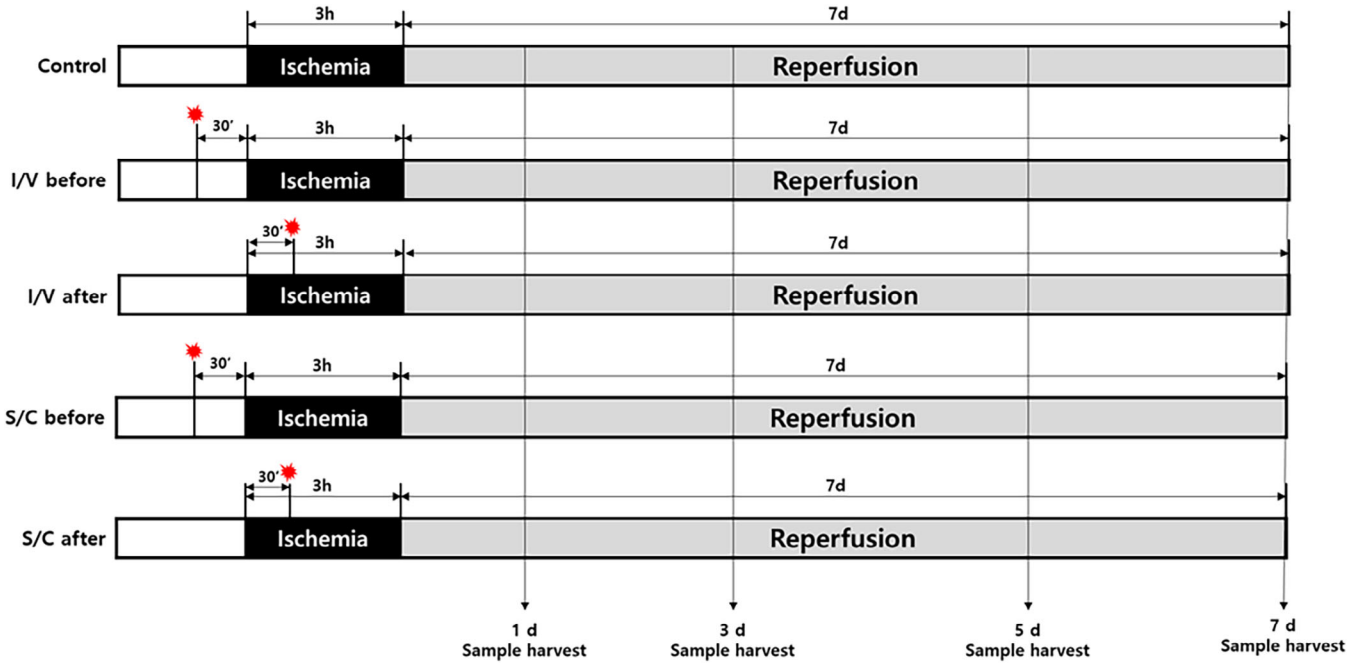

FIGURE 2 Study design.

defatted bovine serum albumin [BSA]) using nitrogen cavitation (Parr Instrument, USA), and then centrifuged at 2000 × *g* for 10 min at 4co to remove cellular debris and nuclei. The supernatant was then centrifuged at 12 000 × *g* for 15 min at 4co to pellet the mitochondria. The pellet was washed twice by suspension in 500 µL SHE buffer followed by centrifugation at 20 000 × *g* for 10 min at 4co. The final pellet was resuspended in 100 µL suspending buffer and kept on ice until use. Isolated mitochondria were quantified by determining protein concentrations using a bicinchoninic acid (BCA) assay.

2.3 | Analysis

The samples of flaps were harvested at intervals of post-surgery days 1, 3, 5, and 7, specifically from the most distal (cephalic) part to the SIE vessels. These collected samples underwent a series of analyses, including histopathological examination, quantitative reverse transcription polymerase chain reaction (RT-qPCR). One animal from each of the five groups was euthanized on these specified days in accordance with recommendations from the ethical committee.

2.4 | Histopathological analysis

Flap tissue samples were harvested and subsequently fixed in a 10% formaldehyde solution for 72 h. Following standard processing and embedding in paraffin blocks, sections of 5 µm thickness were meticulously obtained and affixed onto slides. These prepared tissue slides underwent Hematoxylin and Eosin (H&E) staining according to established protocols to evaluate the pathological changes associated with I/R injury.

2.5 | Immunohistochemistry analysis

The paraffin-embedded slides underwent initial deparaffinized using xylene and subsequent rehydration through various graded ethanol solutions. To quench endogenous peroxidases, a 3% H_2O_2 solution was applied. Antigen retrieval followed, succeeded using goat serum to block non-specific binding. Subsequently, the slides were incubated overnight at 4°C with a primary antibody targeting tumor necrosis factor-alpha (TNF-alpha), caspase 3 (Casp3), superoxide dismutase 1 (SOD1), and hypoxia-inducible factor-1-alpha (HIF-1-alpha), at a 1:50 dilution. After rinsing with phosphate-buffered saline (PBS), a secondary antibody conjugated with horseradish peroxidase was administered. This was followed by PBS washing and staining with 3,3′-diaminobenzidine (DAB), accompanied by hematoxylin counterstaining. The manifestation of the antibody-antigen complex resulted in the development of a brown color. Analysis was conducted employing a light microscope equipped with computer-controlled digital imaging system.

2.6 | RT-qPCR analysis

Total RNA was extracted from tissue samples, and the expression levels of TNF-alpha, interleukin (IL)-1β, IL-6, Casp3, Bcl2, SOD1, and HIF-1-alpha were assessed. The primer sequences utilized for gene amplification in this study are provided in Table 1. Relative quantitative

TABLE 1 The sequences of primer pairs used for amplification of the genes in this study.

Gene	Primer sequence
TNF-α	Forward 5'-AAGGAGGAGAAGTTCCCAAATG-3'
	Reverse 5'-AGAGAACCTGGGAGTAGATAAGG-3'
IL-1ß	Forward 5'-TACCTATGTCTTGCCCGTGGAG-3'
	Reverse 5'-ATCATCCCACGAGTCACAGAGG-3'
IL-6	Forward 5'-AAACGTCCAGAGTGCTAC-3'
	Reverse 5'-CAGCCAGATTTACCTTCA-3'
Casp3	Forward 5'-CCGACTTCCTGTATGCTTACTC-3'
	Reverse 5'-CCAGGGAGAAGGACTCAAATTC-3'
HIF-1	Forward5'-CTCATCCAAGAAGCCCTAACGTGTT-3'
	Reverse 5'-GCTTTCTCTGAGCATTCTGCAAAGC-3'
SOD1	Forward 5'-ATGTGTCCATTGAAGATCGTGTGA-3'
	Reverse 5'-GCTTCCAGCATTTCCAGTCTTTGTA-3'
Bcl2	Forward 5'-AAACGTCCAGAGTGCTAC-3'
	Reverse 5'-CAGCCAGATTTACCTTCA-3'

values were computed based on the Ct values derived from the original qPCR data.

2.7 | Statistical analysis

All statistical analyses were performed using GraphPad Prism software version 9.0 (GraphPad Software, San Diego, CA, USA). The data are presented as the mean ± standard deviation. p-value of less than 0.05 ($p < 0.05$) was considered to indicate statistical significance.

3 | RESULTS

Histological analysis using H&E staining demonstrated a notable decrease in inflammatory cell infiltration in the transplantation group compared to the control, indicating a significant reduction in inflammation (Figure 3). In the TNF-alpha immunohistochemical staining, fewer cells showing a brownish color were observed in the transplantation group in comparison to the control group (Figure 4). Additionally, RT-qPCR analysis revealed statistically significant decrease in TNF-alpha, IL-1β, and IL-6 expression within the transplantation group ("I/V before", "I/V after", "S/C before", "S/C after") compared to the control group (Figure 5). There were no significant differences observed within the transplantation subgroups.

In the Casp3 immunohistochemical staining, a discernible decrease in the number of positively stained cells was observed in the transplantation group compared to the control group (Figure 6). RT-qPCR analysis demonstrated a significant reduction in Casp3 expression across all transplantation subgroups ("I/V before", "I/V after", "S/C before", "S/C after") compared to the control group (Figure 7). Additionally, Bcl2 expression, as measured by RT-qPCR, showed a significant increase in all transplantation groups (Figure 8). No statistically significant differences were observed among the transplantation subgroups.

SOD1 immunohistochemical staining revealed a higher count of brown-colored positive cells in the transplant group compared to the control group (Figure 9). Furthermore, the transplantation groups

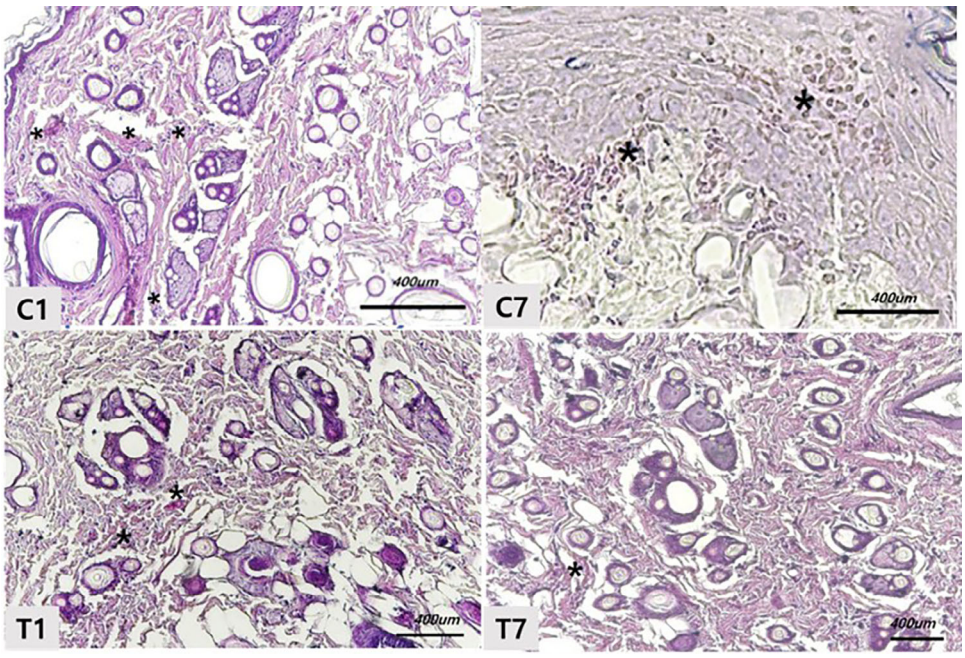

FIGURE 3 Histological analysis of samples stained with Hematoxylin and Eosin. This figure shows the infiltration of inflammatory cells (indicated by asterisks) in the samples. The samples are from the Control group on post-surgery day 1 (C1), the Control group on post-surgery day 7 (C7), the Transplantation group on post-surgery day 1 (T1), and the Transplantation group on post-surgery day 7 (T7).

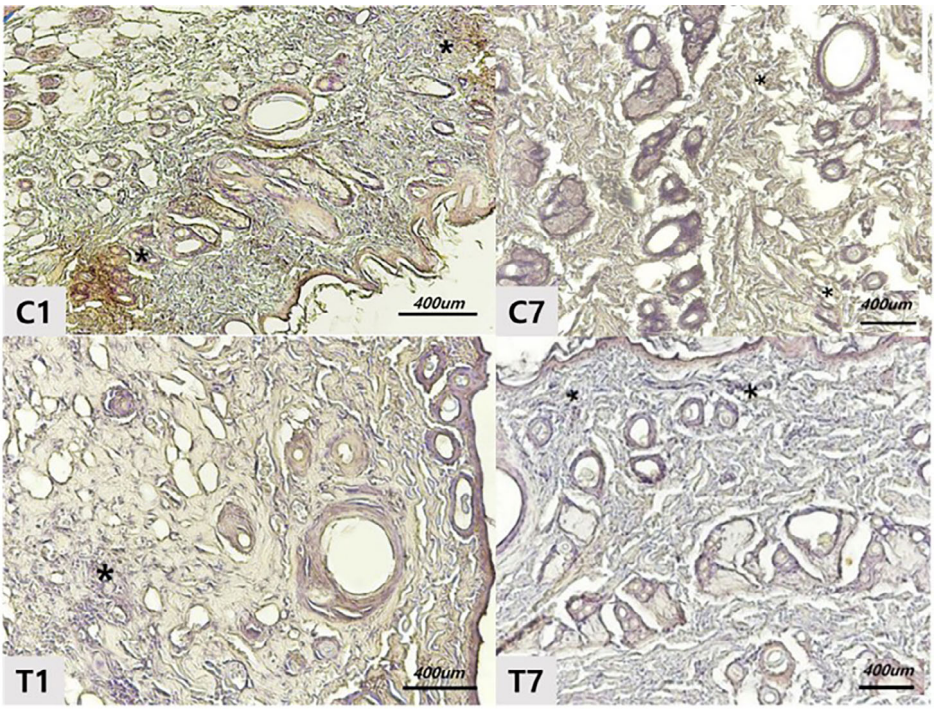

FIGURE 4 TNF-alpha immunohistochemical staining. This figure shows fewer brown-colored TNF-alpha positive cells (indicated by asterisks) in the transplantation group compared to the control group, indicating a reduction in inflammation in the transplanted tissue.

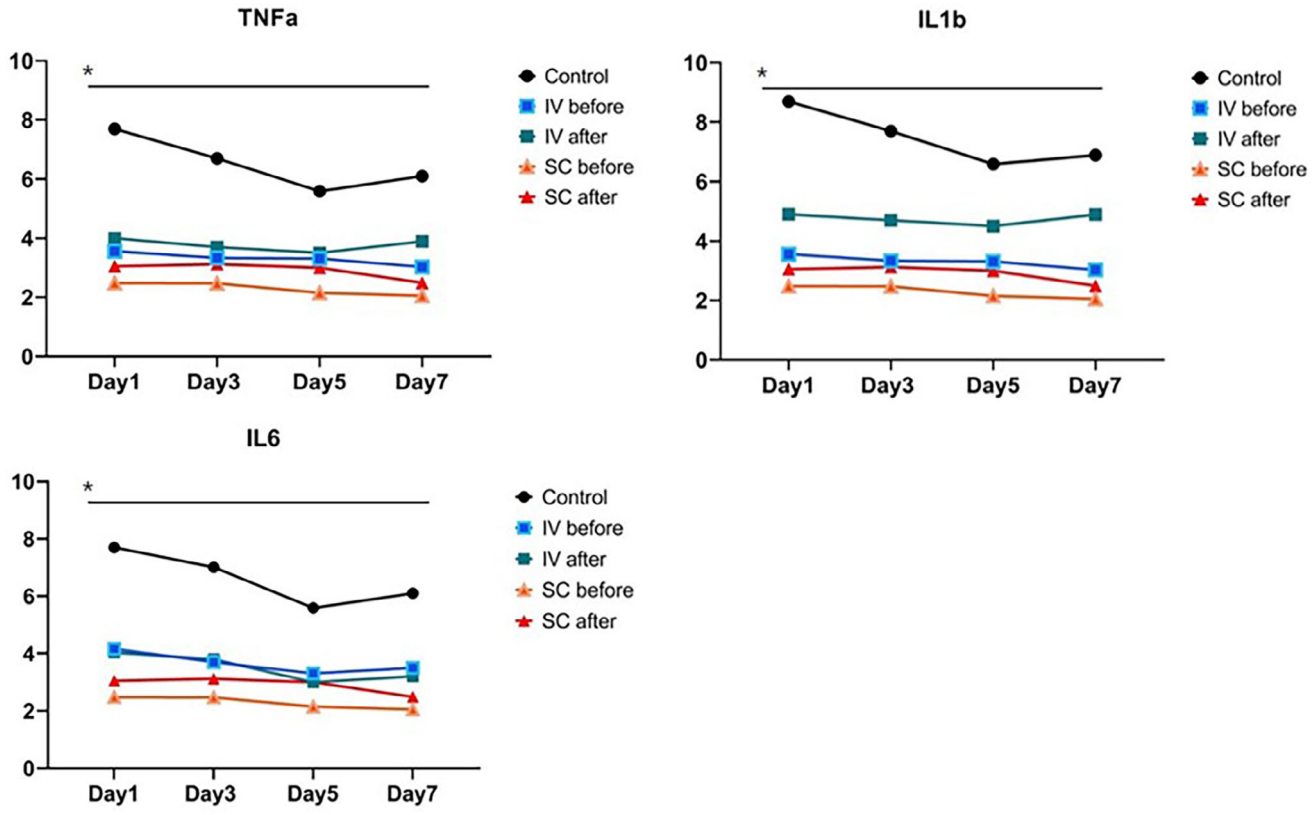

FIGURE 5 RT-qPCR analysis of inflammatory markers. This figure shows the results of an RT-qPCR analysis, which revealed a statistically significant decrease in the expression of TNF-alpha, IL-1β, and IL-6 within the transplantation group ("I/V before", "I/V after", "S/C before", "S/C after") compared to the control group.

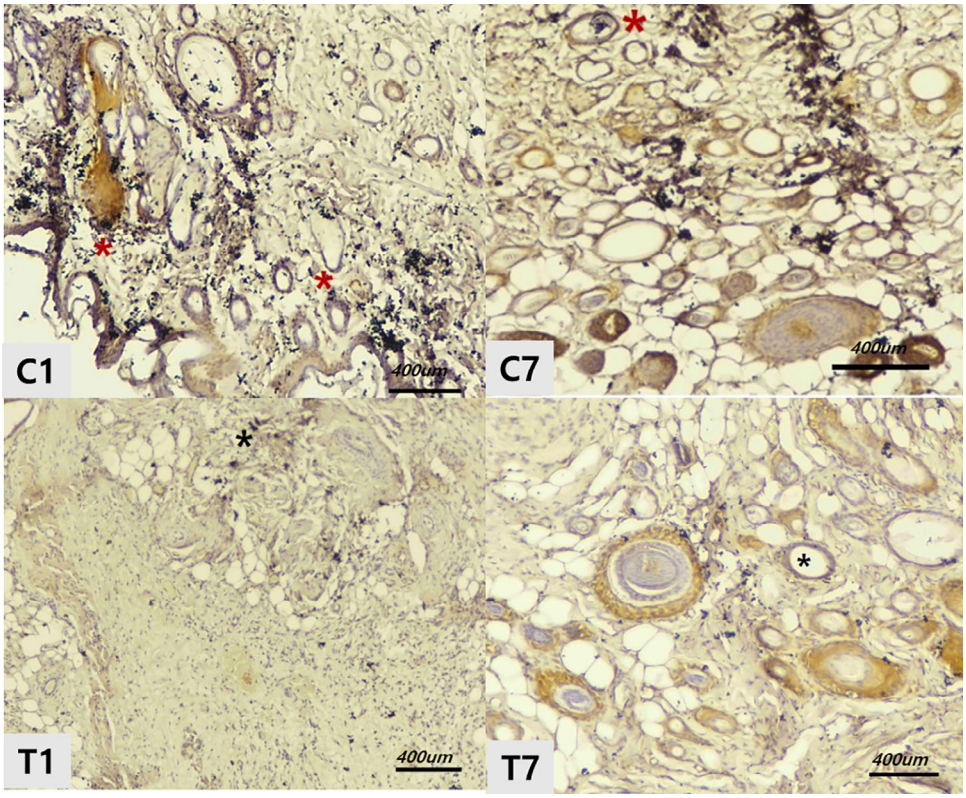

FIGURE 6 Casp3 immunohistochemical staining. A noticeable decrease in the number of positively stained cells (indicated by asterisks) was observed in the transplantation group compared to the control group.

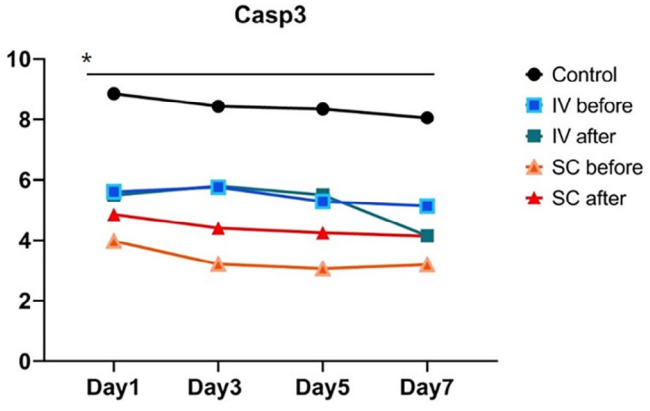

FIGURE 7 RT-qPCR analysis of Casp3 expression. This figure revealed a significant reduction in Casp3 expression across all transplantation subgroups ("I/V before", "I/V after", "S/C before", "S/C after") compared to the control group.

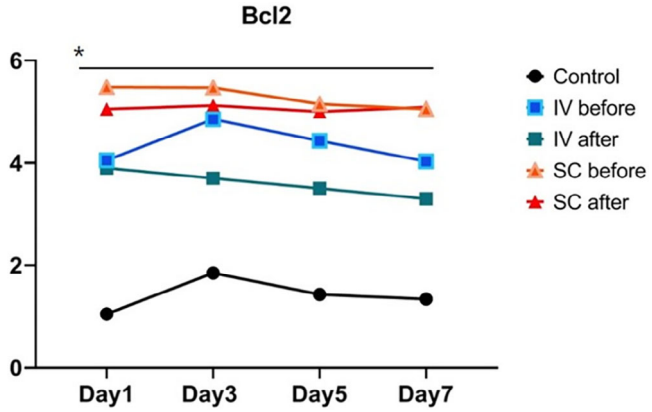

FIGURE 8 RT-qPCR analysis of Bcl2 expression. This figure reveals a statistically significant increase in Bcl2 expression across all transplantation groups, as compared to the control group.

exhibited significantly increased SOD1 expression levels compared to the control group ("I/V before", "I/V after", "S/C before", "S/C after") (Figure 10). No significant differences were observed among the transplantation groups.

HIF-1-alpha immunohistochemical staining revealed a lower count of positive cells in the transplantation group compared to the control group (Figure 11). Additionally, RT-qPCR analysis revealed a significant decrease in HIF-1-alpha levels in all transplantation subgroups compared to the control group ("I/V before", "I/V after", "S/C before", "S/C after") (Figure 12). No statistically significant differences were observed among the transplantation subgroups. Detailed statistical evaluation of the levels is presented in Table 2.

4 | DISCUSSION

This study encompassed a comprehensive assessment of various parameters associated with I/R injury, including tissue histopathology

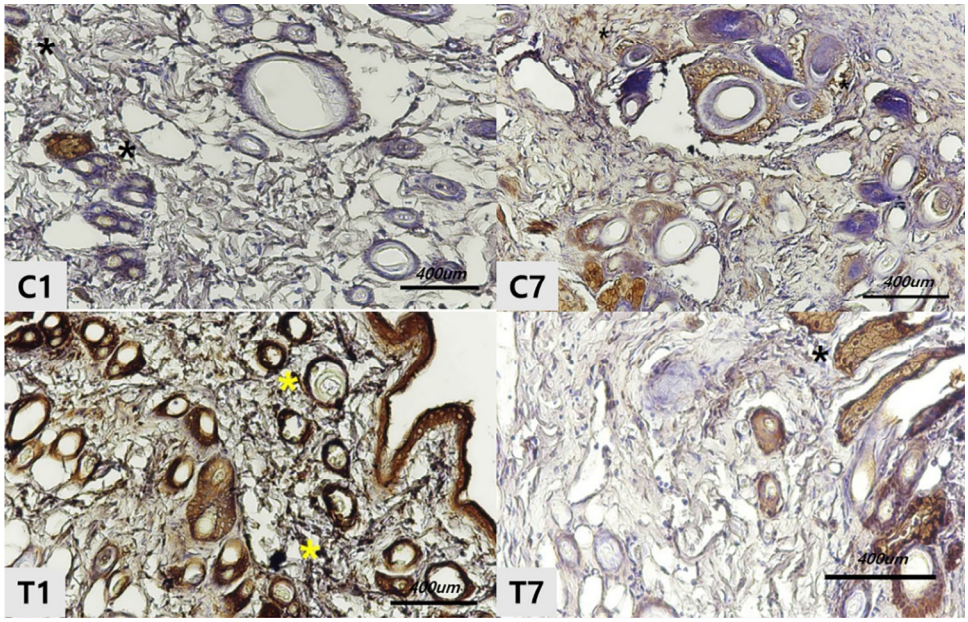

FIGURE 9 SOD1 immunohistochemical staining. This figure shows a higher count of brown-colored positive cells (indicated by asterisks) in the transplantation group compared to the control group.

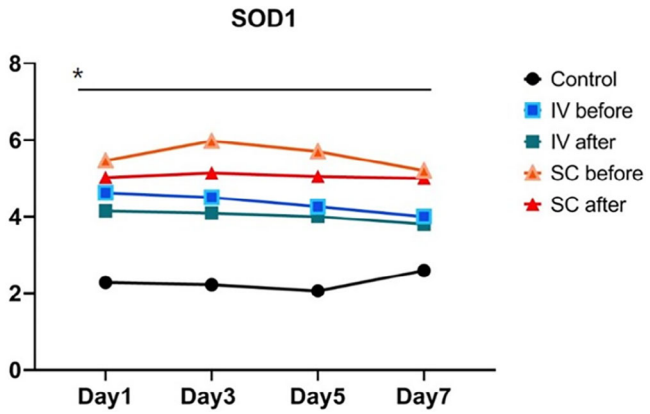

FIGURE 10 RT-qPCR analysis of SOD1 expression. This figure demonstrates that the transplantation groups ("I/V before", "I/V after", "S/C before", "S/C after") exhibited significantly increased SOD1 expression levels compared to the control group.

and markers related to inflammation, oxidative stress, apoptosis, and hypoxia.

Proinflammatory cytokines like TNF-alpha, IL-1β, and IL-6 are central players in the pathology of I/R injury. TNF-alpha, serving as a systemic inflammation signaling protein, initiates diverse immune cell responses that contribute to inflammatory reactions. Similarly, IL-1β and IL-6, produced by leukocytes, exhibit heightened expression with increased TNF-alpha via specific pathways. Studies inhibiting these cytokines have demonstrated reduced tissue damage following I/R injury.[7–10] In the investigation, RT-qPCR analysis revealed decreased expression levels of TNF-alpha, IL-1β, and IL-6 in the transplantation group compared to control group. This reduction in cytokine expression within the transplantation group signifies a notable decrease in inflammation following mitochondrial transplantation.

Casp3, a crucial protease in programmed cell death, becomes activated in response to I/R injury, exacerbating tissue damage by instigating cell death. In animal models, inhibiting Casp3 has proven effective in diminishing apoptosis and fostering tissue recovery following I/R injury.[11–13] Conversely, Bcl2, an anti-apoptotic protein, shields cells from death. Studies showcasing heightened Bcl2 expression have illustrated reduced apoptosis and improved tissue recovery post-I/R injury.[14–16] In the study, decreased Casp3 levels and heightened Bcl2 expression in the transplantation group suggested a decrease in apoptosis post-transplantation of mesenchymal stem cell-derived mitochondria.

SOD1 functions as an essential antioxidant enzyme.[17] Studies in animal models have demonstrated that elevating SOD1 levels effectively mitigates oxidative damage and enhances tissue recovery following I/R injury. In the context of I/R injury, several factors contribute to the decline in SOD1 levels. During ischemia, tissues experience oxygen and nutrient deprivation, prompting the generation of reactive oxygen species (ROS). Upon reperfusion, the sudden reintroduction of oxygen escalates ROS production, overwhelming antioxidant defense mechanisms, including SOD1, leading to its depletion or inactivation. Additionally, the inflammatory response triggered by I/R injury further contributes to the reduction in SOD1 levels. This decrease in SOD1 during I/R injury reflects the impact of oxidative stress and compromised antioxidant capacity.[18,19] In the study, RT-qPCR analysis unveiled heightened SOD1 expression in the transplantation group compared to the control. This finding indicates an increase in SOD1 levels following the transplantation of mesenchymal stem cell-derived mitochondria. Elevated SOD1 expression post-transplantation

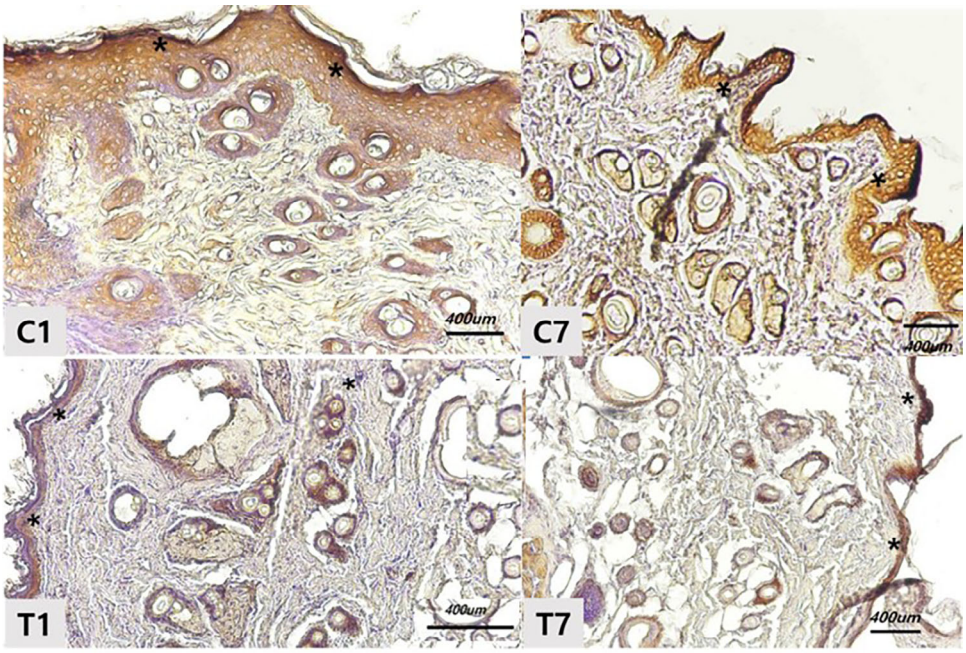

FIGURE 11 HIF-1-alpha immunohistochemical staining: This figure shows a lower count of positive cells in the transplantation group compared to the control group.

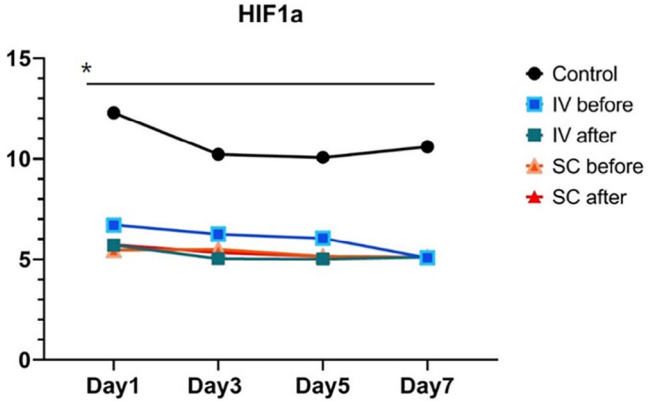

FIGURE 12 RT-qPCR analysis of HIF-1-alpha expression. This figure demonstrates a significant decrease in HIF-1-alpha levels in all transplantation subgroups ("I/V before", "I/V after", "S/C before", "S/C after") compared to the control group.

suggests a potential enhancement in antioxidant defenses, potentially attenuating oxidative stress.

HIF-1-alpha serves as a pivotal transcription factor involved in cellular responses to hypoxia, acting as a critical nuclear regulator that fosters angiogenesis.[20] Under hypoxic conditions, hydroxylation is impeded, leading to the accumulation of HIF-1-alpha.[21,22] In the context of reperfusion following ischemia, HIF-1-alpha is induced. Notably, the study revealed decreased levels of HIF-1-alpha post-transplantation, suggesting a mitigation of hypoxia within the skin flaps.

These results collectively underscore the efficacy of mitochondrial transplantation in reducing I/R-induced inflammation, apoptosis, oxidative stress, and hypoxia within skin flaps, paving the way for potential therapeutic applications in managing such injuries.

While this study offers valuable insights into the potential therapeutic benefits of mesenchymal stem cell-derived mitochondria transplantation in mitigating I/R injury within skin flaps, certain limitations warrant consideration. The investigation primarily focused on a rat model, making direct extrapolation to human scenarios challenging due to inherent biological differences between species. To enhance translatability, future studies in larger animal models, such as pigs, could offer more clinically relevant insights. Moreover, the arbitrary determination of the injected mitochondria quantity and timing might introduce variability in dosage and subsequent effects. Notably, the study did not demonstrate discernible differences between the two distinct delivery methods (intravenous and subcutaneous) and timing (before and after). However, further research employing broader markers, diverse models, and detailed mechanistic studies is necessary. Larger sample sizes and extended observation periods are needed to comprehensively assess these methods' comparative effectiveness. Recent systematic review papers on mitochondrial transplantation consistently reported positive outcomes. However, discrepancies in the species, target organs, source of mitochondria, transplantation techniques, dosage, and timing were evident across studies, indicating a lack of uniformity in these critical parameters.[23] Integrating these findings highlights the need for standardized approaches in dosage, delivery methods, and timing for more robust and comparable results in future research. Lastly, the absence of a sham group without I/R injury or only buffer injected control group limits the comprehensive understanding of the exclusive impact of transplantation alone.

TABLE 2 Mean levels of each gene expression in each group.

Groups	Control	I/V before	I/V after	S/C before	S/C after	P*
TNF-alpha	6.52 ± 0.48	3.31 ± 0.76	3.97 ± 0.79	2.28 ± 0.73	2.91 ± 0.84	<0.05
IL-1ß	7.47 ± 0.76	3.30 ± 0.78	4.74 ± 0.85	2.28 ± 0.76	2.91 ± 0.84	<0.05
IL-6	6.77 ± 0.42	3.65 ± 0.49	3.57 ± 0.25	2.48 ± 0.51	2.85 ± 0.52	<0.05
Casp3	8.41 ± 0.53	5.44 ± 0.24	5.23 ± 0.56	3.35 ± 0.22	4.41 + 0.07	<0.05
Bcl2	1.41 ± 0.42	4.34 ± 0.15	4.34 ± 0.15	5.28 ± 0.37	5.06 ± 0.30	<0.05
SOD1	2.28 ± 0.12	4.34 ± 0.43	4.01 ± 0.34	5.58 ± 0.51	5.05 ± 0.25	<0.05
HIF-1-alpha	10.78 ± 0.53	6.01 ± 0.89	5.21 ± 0.69	5.19 ± 0.47	5.32 + 0.78	<0.05

P*: Control group vs. Each transplantation subgroup
No statistically significant differences were observed among transplantation subgroups.

5 | CONCLUSION

This comprehensive study underscores the multifaceted impact of umbilical cord mesenchymal stem cell-derived mitochondria transplantation in mitigating I/R injury. Collectively, above findings suggest that umbilical cord mesenchymal stem cell-derived mitochondria transplantation holds promise as a therapeutic strategy for ameliorating I/R injury. By modulating inflammation, apoptosis, and oxidative stress responses, it paves the way for improved outcomes involving I/R injury within skin flaps.

ACKNOWLEDGMENTS

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards of interest. The study was approved by the Institutional Review Board of Soonchunhyang University Bucheon Hospital (IRB SCHBCA2022-07) and performed in accordance with the principles of the Declaration of Helsinki.

INFORMED CONSENT

For this type of study, all the patients had signed the informed consent.

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