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Tissue integrity and healing response in hypoestrogenic animal model treated by mesh implantation with addition of mesenchymal stem cell secretome

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ABSTRACT

Background: Pelvic organ prolapse increases in prevalence and incidence in older women and hypoestrogenic conditions. Treatment with native tissue surgery has a fairly high recurrence rate. Mesh-augmented surgery is one of the most promising treatments for pelvic organ prolapse, with high effectiveness and low recurrence. Mesh-augmented surgery has a side effect of tissue erosion. The addition of secretome is expected to improve tissue integrity and reduce tissue erosion.

Aim: This study aimed to investigate the effect of adding the umbilical cord mesenchymal stem cell (UC-MSC) secretome on preventing tissue inflammatory responses, improving tissue integrity, and accelerating wound healing.

Methods: A total of 32 female New Zealand white rabbit hypoestrogenic models were divided into two groups: the control group with normal mesh and the secretome group with artificial mesh. Hypoestrogenic models were created using the bilateral ovariectomy method. Mesh implantation was performed using a surgical method on hypoestrogenic rabbits. The animals were euthanized on days 7, 14, 28, and 90 after mesh implantation. Histopathology parameters included angiogenesis formation, fibroblast number, and collagen deposition area.

Result: The results of this study showed that the number of angiogenesis, fibroblast, and collagen deposition data in the secretome group showed higher significantly ($p < 0.05$) than those in the control group on days 7, 14, 28, and 90 post mesh implantation. The formation of new blood vessels (angiogenesis) in the secretome group demonstrated a mean value of 9.81 ± 2.2 compared to 0.37 ± 0.03 in the control. The number of fibroblasts in the secretome group averaged 151.00 ± 8.14, in contrast to 34.00 ± 13.37 in the control group. Collagen formation in the secretome group was also higher, with a mean value of 80.02 ± 6.71 compared to 59.49 ± 4.61 in the control group over 90 days of observation.

Conclusion: The administration of secretomes from UC-MSC improved tissue integrity and accelerated wound healing.

Keywords: Hypoestrogenic, Angiogenesis, Mesh, Prolapse, Secretome.

Introduction

Uterine prolapse is a form of pelvic organ prolapse caused by weakness of the ligament and supporting fascia, leading to the exit of the uterus through the vagina. The prevalence of uterine prolapse varies depending on the population studied and the diagnostic methods employed; however, it is estimated to affect approximately 3%–6% of women worldwide in clinically diagnosed cases. When including milder and asymptomatic cases, the prevalence may increase to around 30%–50%, particularly among women over the age of 50 years. In the United States, approximately 11%–19% of women are expected to undergo surgery for prolapse or incontinence at some point in their lives. Pelvic organ prolapse is a frequent medical condition whose prevalence and incidence increase with age and hypoestrogenic conditions that result in weakness of the ligaments and fascia supporting the uterus. Estrogen plays a crucial role in the wound-healing process across all stages, including hemostasis/inflammation, proliferation, and remodeling. It helps by reducing the size of the wound, enhancing collagen deposition by regulating the levels of collagen I and III during the remodeling phase, and strengthening the tissue. Estrogen can also promote angiogenesis and regeneration by increasing the rate of epidermal cell

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mitosis. Low estrogen levels inhibit the wound-healing process.

Pelvic organ prolapse treatment with native tissue surgery has a short-term anatomical repair rate of 35%–72% and recurrence is quite high, reaching 30%, due to the results of repair with native tissue showing that there are still weaknesses in the supporting connective tissue that have been repaired (Van Geelen and Dwyer, 2013). Mesh treatment is one of the most promising treatments for pelvic organ prolapse. The augmentation of prolapsed tissue with mesh-augmented repair is a reconstructive technique that provides satisfactory results, with an effectiveness rate of 76%–96% and a recurrence rate of 7.4% (Skala *et al.*, 2011). Mesh-augmented surgery has high efficacy and low recurrence. However, mesh-related complications leading to tissue erosion have been reported in 55% of cases, which is thought to be due to suboptimal wound healing and tissue integration with the mesh (Moen *et al.*, 2014). The use of mesh has decreased dramatically with the U.S. Food and Drug Administration (FDA) warning, regarding postoperative complications, particularly mesh erosion/extrusion. In 2011, the FDA reported several complications arising after mesh insertion in pelvic organ prolapse repair, including mesh erosion, infection, bleeding, urinary tract problems, and organ perforation (Mancuso *et al.*, 2019).

To address the tissue damage associated with mesh implantation in pelvic organ prolapse, novel therapeutic approaches are being explored to improve outcomes. One promising strategy involves integrating reconstructive surgery with cell-based therapies, specifically leveraging stem cells because of their regenerative potential. Recent advancements in stem cell research have led to the use of the stem cell secretome, which contains bioactive molecules secreted by stem cells as progenitor agents for tissue repair and regeneration. In this study, the application of the stem cell secretome represents an innovative approach in preliminary trials aimed at combining stem cell-derived factors with reconstructive surgical techniques to enhance pelvic tissue integrity and function.

The mesenchymal stem cell secretome contains a number of chemokine and cytokine growth factors, such as interleukin-6 and interleukin-8 (IL-6, IL-8), proteases and protease inhibitors, such as matrix metalloproteinase (MMP)-1 and 2, and extracellular matrix (ECM) molecules that assist in cell regeneration to accelerate the wound-healing process (Dilogo *et al.*, 2020). Stem cells also provide growth factors that stimulate endothelial growth factors to accelerate angiogenesis (Amani *et al.*, 2019). In another field, the addition of stem cells accelerates muscle tissue regeneration by increasing the proliferation of myocyte cells (Bakhtiary *et al.*, 2021). The addition of mesenchymal stem cell secretomes to the mesh can improve tissue integration and prevent excessive tissue inflammatory responses, thereby minimizing tissue erosion. This study aimed to analyze the inflammatory response and tissue healing after mesh insertion with the addition of umbilical cord mesenchymal stem cell (UC-MSC) secretome in hypoestrogenic rabbit models.

Materials and Methods

Ethical approval

All procedures in this study were approved by the Animal Ethics Commission of the School of Veterinary Medicine and Biomedical Sciences, IPB University (certificate number: 002/KEH/SKE/VI/2022.

Materials

In this study, we used stem cell secretome as the primary investigational material, with female New Zealand White rabbits serving as the animal model. The experimental procedures involved the use of an incubator (Labline, JPN), centrifuge (Labogene 1580R, JPN), Laminar Chamber (Thermo Scientific, JPN), Microtome (Leica, JPN), surgical instruments (Reinz, ITA), and sutures using Prolene® 3.0. Anesthesia was administered using a combination of ketamine (KET-A 100, PER) and xylazine (Xyla 20, USA) and EUTHASOL® (pentobarbital sodium and phenytoin sodium). Additionally, a mesh implant was used along with hematoxylin-eosin (HE) staining and Masson's trichrome staining kits for histological analysis.

Secretome preparation

The secretome was prepared at the Stem Cell and Tissue Engineering Research Cluster, Indonesian Medical Education and Research Institute, University of Indonesia, following standardized laboratory protocols for extraction from UC-MSCs. Five passage UC-MSCs were cultured in six 25 cm² flasks, each containing 5 ml of complete medium, with medium changes conducted every 2–3 days. The cell cultures were maintained in a controlled environment, with an incubator set to 37°C and 5% CO_2 to ensure optimal cell growth. When cell confluency reached 80%–90%, the conditioned culture medium was harvested for secretome processing. A total volume of 30 ml of conditioned medium was collected from the six flasks. The conditioned medium was centrifuged at 3,500 rpm for 30 minutes to separate the cellular debris. The supernatant was then sequentially filtered through 0.45 and 0.22 µm filters to remove particulates, resulting in a purified secretome for downstream applications.

Modeling a hypoestrogenic animal

This study used 32 female New Zealand white rabbits *(Oryctolagus cuniculus*) at 1 year of age, with body weights of 3.0–3.5 kg. Modeling of hypoestrogenic animal models was carried out using the bilateral ovariectomy method. The hypoestrogenic conditions were determined by measuring blood estradiol levels before ovariectomy surgery (day 0) as a baseline and 30-day post-ovariectomy. The results of the examination of estradiol levels showed that the rabbit models experienced hypoestrogenic conditions on the 30th day after ovariectomy, marked by a decrease in estradiol

levels of more than 50% of those before ovariectomy. In addition, vaginal cytology was performed to ensure that the animal was no longer in the estrus phase. Vaginal cytology on day 30 revealed only small and large nucleated epithelial cells. Cornified epithelial cells were not observed. The animal model was created as described previously (Dewi *et al.*, 2024).

Mesh implantation

Hypoestrogenic rabbits were divided into two groups: the control and secretome groups. Each group contains 16 animals. Before mesh implantation, the animal models were anesthetized using a combination of ketamine (10 mg/kg BW and Xylazine 3 mg/kg BW) via intramuscular injection. After anesthesia, a 2-cm transversal incision was made on the anterior vaginal wall, followed by tissue dissection of 1.5–2.0 cm laterally and 3.0–3.5 cm longitudinally to the rectovaginal fascia. Mesh implantation was performed in the submucosal layer of the anterior vagina. All mesh corners were fixed to the tissue using prolene® 3.0 suture. In the treatment group, the mesh was supplemented with secretome. The peritoneum was closed with simple sutures, and the subcutaneous layer was sutured with continuous sutures using prolene® 3.0. Skin incisions were sutured using silk braided® 3.0. The subjects were followed for 90 days, and macroscopic and microscopic analysis was performed on days 7, 14, 3, and 90 post-mesh implantations. Animal models were euthanized with EUTHASOL® (pentobarbital sodium and phenytoin sodium) at a dose of 0.2 ml/kg body weight. We conducted euthanasia according to the rules of the AVMA Guidelines for the Euthanasia of Animals (AVMA, 2020).

Histopathological and immunohistochemical analysis

The samples were fixed in 10% neutral buffered formalin and followed in tissue processed by paraffin-embedded methods. A 5-µm-thick tissue section was stained by HE. HE staining was employed to examine angiogenesis (formation of new blood vessels) and fibroblast proliferation during the observation. Angiogenesis and fibroblast numbers were counted manually in five fields of view in the mesh implantation area.

Masson's trichrome staining was used to observe collagen deposition in tissue with mesh implantation, as previously described. The aniline blue expression related to collagen was measured by calculating the proportion area and other objects using ImageJ (www.imagej.net).

Data analysis

The data is statistically processed by comparing Control and Secretome group with paired t-test method and visualization graph was performed in R software ver. 4.4.0 (www.r-project.org).

Results

Measurement of angiogenesis and fibroblast cells

The measurement of angiogenesis volume is presented in Figure 1. Histopathological staining results are shown in Figure 2. The secretome group had a

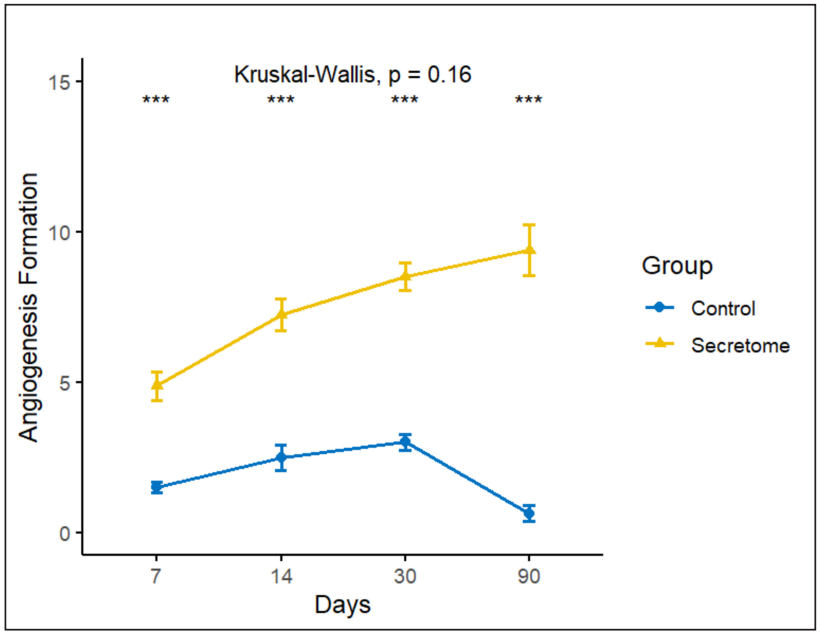

Fig. 1. Number of angiogenesis formation in the experimental rabbit after 90 days of observation in the control and secretome groups. Asterisks (*) imply significance value with $p \leq 0.05$ (**$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$). Data represent mean ± SD.

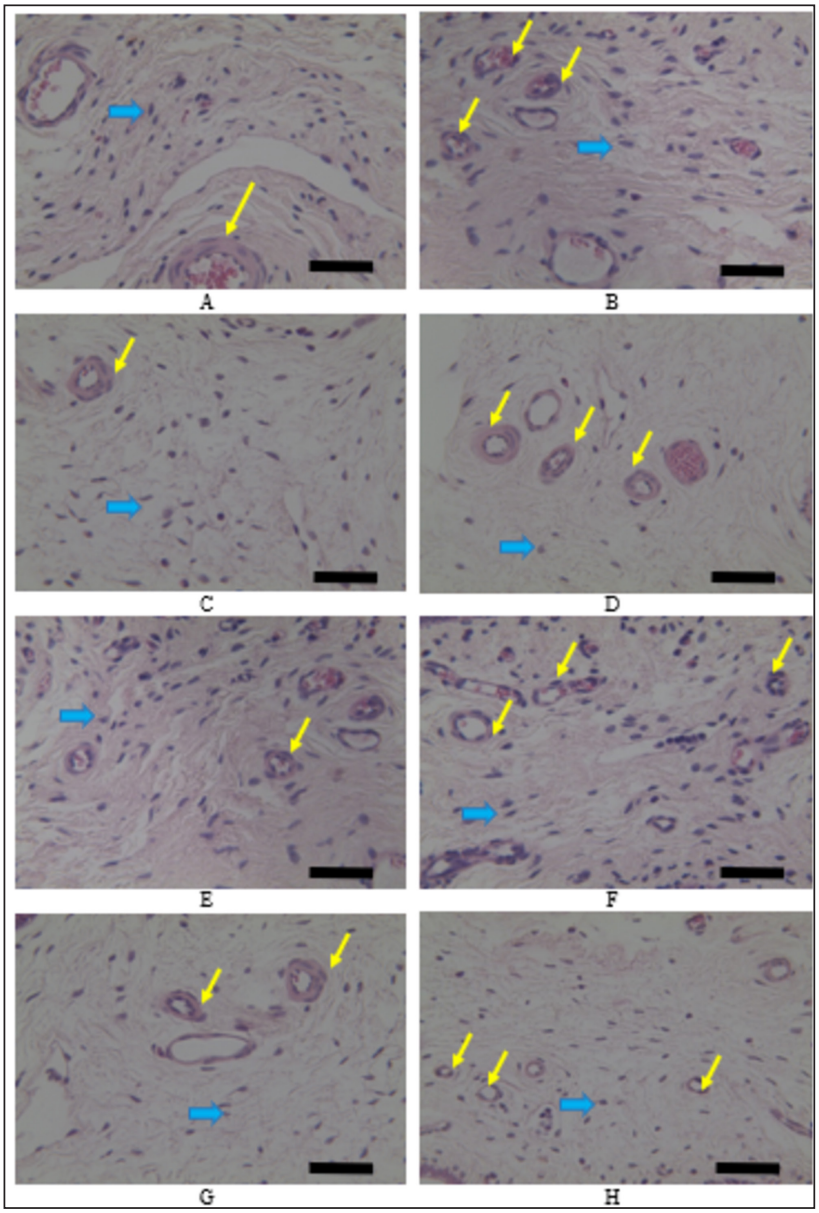

Fig. 2. Microscopic observation of angiogenesis and fibroblast cells using HE staining and observed using microscope with 100× magnify. A. Control groups on day 7, B. Secretome groups on day 7, C. Control group on day 14, D. Secretome groups on day 14, E. Control groups on day 30, F. Secretome groups on day 30, G. Control groups on day 90, H. Secretome groups on day 90. The yellow arrow indicates new vascular blood in the tissue, and the blue arrow indicates fibroblast cells in the tissue. The scale bar equals with the 100 µm.

significantly higher all-day observation rate than the control group ($p < 0.05$). Angiogenesis increased consistently in the secretome group until the end of observation. Based on Figure 1, the formation of new blood vessels experienced an increasing trend during the observation period, in contrast to the control group, which experienced a decrease on day 90.

Fibroblast cell counts are presented in Table 1. In line with the angiogenesis data, the measurement of the number of fibroblasts showed that the secretome group had a higher number of fibroblasts than the control group. The results of measuring the number of fibroblasts showed a significant difference between the secretome and control groups on all days of observation (Fig. 2). The number of fibroblasts was higher in the

Table 1. Average number of fibroblasts in the control and secretome groups during the observation.

Groups	Days			
	7	14	28	90
Control	26.00 ± 9.09[a]	40.00 ± 8.79[a]	56.00 ± 14.27[a]	34.00 ± 13.37[a]
Secretome	50.87 ± 11.07[b]	78.37 ± 12.07[b]	138.37 ± 25.60[b]	151.00 ± 8.14[b]

The superscripted letters indicate significant differences ($p < 0.05$) at the 95% confidence level.

Table 2. Average area of collagen deposition between the control and secretome groups during observation.

Groups	Days			
	7	14	28	90
Control	29.33 ± 7.59[a]	33.37 ± 10.21[a]	40.04 ± 9.38[a]	59.49 ± 4.61[a]
Secretome	43.89 ± 8.19[b]	49.95 ± 9.71[b]	61.75 ± 6.15[b]	80.02 ± 6.71[b]

The superscripted letters indicate significant differences ($p < 0.05$) at the 95% confidence level.

secretome group and increased during the observation day, in contrast to the control group, which decreased on day 90. The observed increase in the formation of angiogenesis and the elevated number of fibroblasts indicate promising outcomes during tissue healing. The formation of new blood vessels is critical because it ensures an adequate supply of nutrients and oxygen to the damaged tissue, thereby facilitating repair and regeneration. Fibroblasts are essential for maintaining tissue integrity because they contribute to the ECM and play pivotal roles in the tissue structural framework. Their presence is vital for stabilizing tissue architecture and the overall healing process.

Measurement of collagen deposition

The results of collagen deposition are presented in Table 2. The results of collagen deposition measurements showed that collagen deposition increased in both research groups as the research observation time progressed; however, the area number of collagen deposition in the secretome group was significantly higher than that in the control group at all observation days ($p < 0.05$) (Fig. 3). The observed increase in collagen deposition suggests that secretome administration enhances tissue stability. Collagen is a fundamental component of the ECM and plays a critical role in the healing process by providing structural support and contributing to the maintenance of tissue stability and elasticity.

Discussion

The evaluation of secretome administration during mesh implantation for pelvic organ prolapse in this study yielded favorable outcomes. The assessment focused on tissue healing and integrity using the parameter of increased neovascularization (angiogenesis). This parameter is an indicator of ongoing tissue repair. The study found that the secretome treatment group exhibited a significantly higher number of angiogenesis events compared with the control group. The formation of new blood vessels (angiogenesis) in the secretome group demonstrated a mean value of 9.81 ± 2.2 compared to 0.37 ± 0.03 in the control. These findings demonstrate markedly improved outcomes compared with those reported by Mancuso *et al.* (2019). This effect is attributed to the mesenchymal stem cell secretome containing a number of chemokine and cytokine growth factors such as IL-6, IL-8, proteases and protease inhibitors such as MMP -1 and 2, and ECM molecules that will assist in cell regeneration so as to improve tissue integration, prevent excess tissue inflammatory response, and accelerate the wound-healing process (Dilogo *et al.*, 2020). Based on the refference, it is necessary to test and measure in living tissue the ability of stem cell secretome tolerance when applied as a medicinal preparation. A previous study proved that secretomes have therapeutic potential (Hacker *et al.*, 2021). The study of secretomes, or secretomes, has significant implications for diagnostics and therapeutics. The secretome reflects the state of cells and tissues and can serve as a source of biomarkers for diseases such as cancer, cardiovascular diseases, and neurodegenerative disorders (Jerard *et al.*, 2023). Secretomes play a significant role in the healing process because they comprise various signaling molecules, enzymes, growth factors, and ECM components that facilitate tissue repair and regeneration. The healing process generally involves several stages: hemostasis, inflammation, proliferation, and remodeling (Damayanti *et al.*, 2021; Hacker *et al.*, 2021; Jammes *et al.*, 2023; Tilotta *et al.*, 2023). The secretome effect is observed throughout these stages, with different cell types contributing to the secreted factors that drive healing. In the wound-healing process, the administration of secretome helps accelerate healing because secretome plays a role in stimulating the migration of the number of white blood cells, especially macrophages, to the wounded

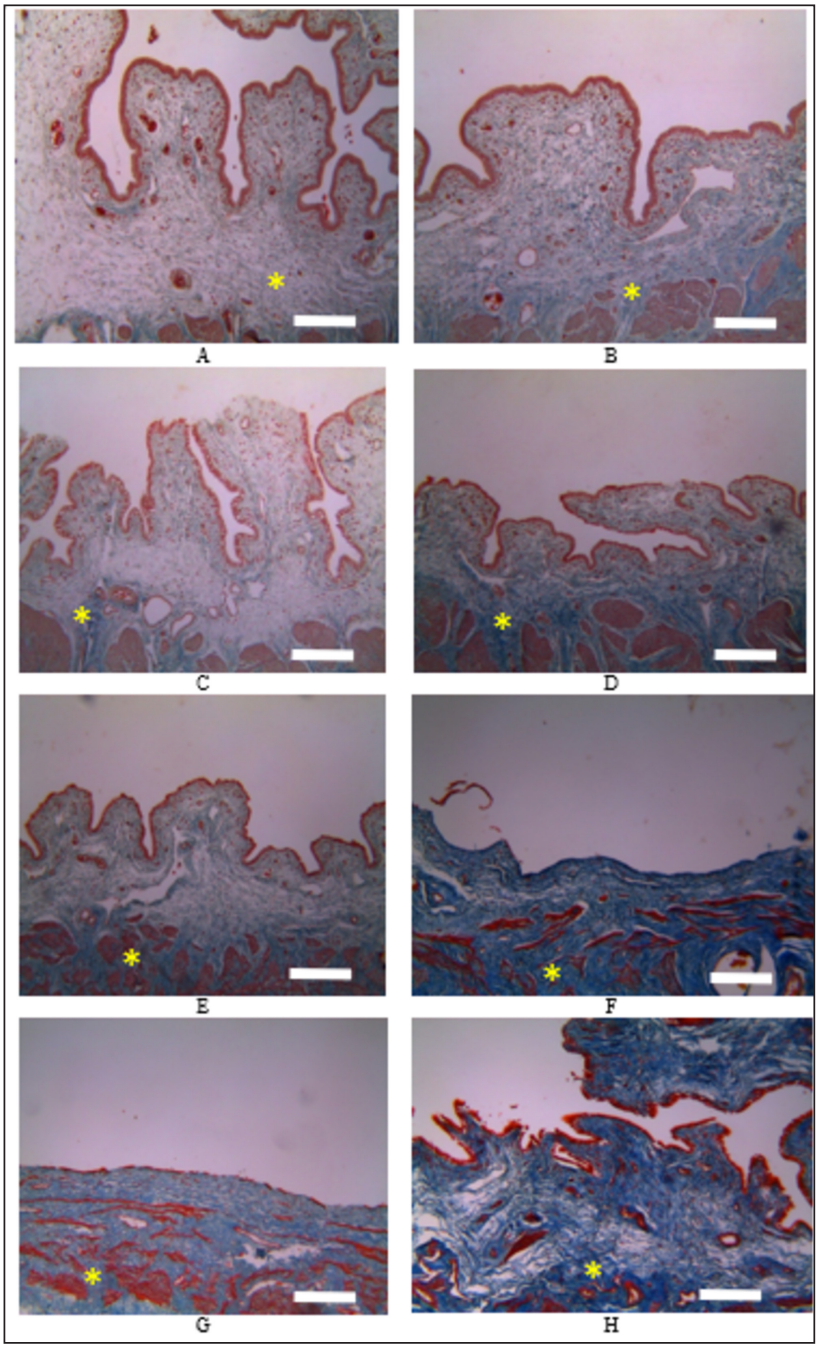

Fig. 3. Microscopic observation of collagen area deposition using Masson's trichrome staining and observed using microscope with 100× magnify. A. Control groups on day 7, B. Secretome groups on day 7, C. Control group on day 14, D. Secretome groups on day 14, E. Control groups on day 30, F. Secretome groups on day 30, G. Control groups on day 90, H. Secretome groups on day 90. Asterisk (*) refers to the area of collagen deposition. The scale bar equals with the 100 µm.

area, so that the number of macrophages increases, the secretion of growth factors, such as epidermal growth factor (EGF), fibroblast growth factor (FGF), and vascular endothelial growth factor (VEGF), also increases. These growth factors stimulate the proliferation (cell division) and migration (cell movement) of cells involved in the healing process. The proliferation and migration of these cells are

important for the formation of new tissue and wound closure (Shi *et al.*, 2018; Porro *et al.*, 2020).

Tissue integrity refers to the structural and functional stability of tissues, ensuring that they can perform their physiological roles effectively (Hacker *et al.*, 2021). The secretome influences tissue integrity through various mechanisms, including promoting cellular communication, supporting the ECM, modulating immune responses, and facilitating repair and regeneration processes. The components of the secretome involved in tissue integrity are ECM proteins (Maacha *et al.*, 2020; Sandonà *et al.*, 2021).

The secretome treatment group exhibited a significantly higher fibroblast proliferation rate compared with the control group. This difference is attributed to the secretion of proteins such as collagen, fibronectin, laminin, and elastin, which form the structural framework of the ECM. These components provide mechanical support and elasticity to tissues and are crucial for maintaining tissue architecture. Growth factor molecules such as transforming growth factor-beta (TGF-β), FGFs, and EGF are involved in cell proliferation, differentiation, and migration (Maacha *et al.*, 2020). These growth factors are vital for tissue maintenance and repair. Enzymes such as MMPs degrade ECM components, which are crucial for tissue remodeling and repair. The activity of these enzymes is tightly regulated by tissue inhibitors of metalloproteinases (TIMPs) to prevent excessive degradation and maintain tissue integrity (Ahangar *et al.*, 2020; Maacha *et al.*, 2020; Gwam *et al.*, 2021; Jammes *et al.*, 2023).

The mechanisms by which the secretome maintains tissue integrity include ECM maintenance and remodeling (Cases-Perera *et al.*, 2022). The secretome contributes to the dynamic balance of ECM synthesis and degradation, ensuring that tissues maintain their structural properties. For instance, collagen and other matrix proteins strengthen the ECM, whereas MMPs and TIMPs regulate the remodeling and turnover of ECM components (Maacha *et al.*, 2020). Cell communication and coordination, growth factors, and cytokines in the secretome facilitate communication between cells, coordinating responses to physiological and pathological stimuli. This communication is essential for processes such as tissue development, repair, and immune responses (Liu *et al.*, 2020; Maacha *et al.*, 2020; Jammes *et al.*, 2023). Angiogenesis, the formation of new blood vessels, is crucial for supplying nutrients and oxygen to tissues, especially during repair and regeneration. The secretome promotes angiogenesis through factors such as VEGF and angiogenesis, ensuring adequate blood supply and supporting tissue health (Ahangar *et al.*, 2020; Maacha *et al.*, 2020; Porro *et al.*, 2020; Sears *et al.*, 2020).

Secretome plays many roles in collagen deposition: the first role in collagen synthesis. Secretome can stimulate cells to produce TGF-β to stimulate collagen synthesis by activating fibroblasts and promoting the expression of collagen genes (Cifuentes *et al.*, 2021; Reyes-Ramos *et al.*, 2021). In addition, it enhances the production of other ECM components. FGFs can promote fibroblast proliferation and collagen production, thereby aiding tissue repair and ECM maintenance (Cifuentes *et al.*, 2021). The platelet-derived growth factor (PDGF) function enhances fibroblast activity and collagen production, particularly in wound healing. Certain interleukins (ILs), such as IL-6 and IL-10, can influence collagen production by modulating fibroblast activity and ECM composition (Basalova *et al*., 2020; Zhou *et al*., 2021).

In collagen maturation, the secretome plays a role in many stages of maturation. In post-translational modifications, secretomes play a role in hydroxylation and glycosylation, and they are essential for collagen stability and functionality (García de Vinuesa *et al.*, 2016; Ahangar *et al.*, 2020; Sandonà *et al.*, 2021). The secretome provides enzymes and cofactors necessary for these modifications, such as prolyl, lysyl hydroxylase, and proteolytic enzymes. The secretome also plays a role in MMPs and is involved in the degradation of ECM components, including collagen. MMPs such as MMP-1 (collagenase) facilitate the breakdown of mature collagen fibers, which is crucial for tissue remodeling and repair. The secretome also participates in collagenase enzyme synthesis. Collagenases are specific MMPs that target and degrade collagen, allowing for remodeling and the replacement of old or damaged collagen. In TIMPs, collagen can regulate MMP activity, ensuring a balance between collagen degradation and synthesis. Proper regulation is essential for maintaining ECM homeostasis and preventing excessive tissue damage or fibrosis (Sears *et al.*, 2020; Damayanti *et al.*, 2021; Zhou *et al.*, 2021).

The secretome stimulates the formation of growth factors. Many growth factors during wound healing are closely related to wound repair, one of which is FGF (Wilkinson *et al.*, 2017). FGF is a polypeptide growth factor with diverse biological functions. Secreted FGF can be classified into two categories: classical FGF (also known as paracrine FGF) and endocrine FGF. Apart from their conventional role in regulating cell proliferation and differentiation, fibroblasts serve as more potent angiogenesis factors than PDGF and VEGF (Porro *et al.*, 2020). These growth factors may be related to angiogenesis and fibroblast stimulation in secretome-treated wounds.

The results showed that the stem cell secretome was able to significantly induce collagen formation compared with the control group. In line with other studies, collagen levels were significantly higher in the secretome-treated group than in the control group (Wilkinson *et al.*, 2017; Cifuentes *et al.*, 2021; Ajit *et al.*, 2023). The secretome contains various growth factors and cytokines that stimulate collagen production by cells such as fibroblasts. Tissue healing requires

collagen synthesis, which is an important part of cell growth (Ravishankar *et al.*, 2018). The secretome may also contain transcription factors or regulatory signals, such as specificity protein-1 and activator protein-1 can bind to the promoters of collagen genes and regulate their expression (Reyes-Ramos *et al.*, 2021). When a wound occurs, fibroblasts produce new collagen to help repair the tissue. During the early phase of wound healing, the synthesis of Type III collagen is most prominent, accompanied by the presence of inflammatory cells. Type III collagen is replaced by type I collagen, which has a stronger and more stable linking ability that increases tensile strength after implantation (Gonzalez *et al.*, 2016).

Conclusion

The administration of an UC-MSC secretome has shown promising potential to enhance tissue integrity and accelerate wound healing. The regenerative properties observed in this study suggest that UC-MSC secretomes could serve as valuable therapeutic tools, particularly in conditions characterized by compromised tissue repair, such as pelvic organ prolapse, chronic wounds, and tissue injuries resulting from surgery.

Further research is warranted to refine and optimize secretome formulations, including the identification of specific growth factors and cytokines responsible for these regenerative effects. Advanced studies involving larger clinical trials are essential to confirm the safety, efficacy, and long-term benefits. Additionally, exploring delivery methods such as injectable hydrogels, biocompatible scaffolds, and targeted release systems could enhance the therapeutic reach of the UC-MSC secretome, making it adaptable to diverse clinical needs.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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Authors' contributions

TITD: Study design, data collection during research, data interpretation, and manuscript preparation. DN: supervised the research, interpreted the data, drafted, and revised the manuscript. BPP: interpreted the data of histopathology, drafted, and revised the manuscript. GNT: supervised the research, drafted, and revised the manuscript. MSB: data analyzed, drafted, and revised the manuscript. All authors have read, reviewed, and approved the final manuscript.

Data availability

All data were provided in the manuscript.

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