PLA/Wax Scaffolds Enhance Wharton's Jelly-derived Mesenchymal Stem Cells for Blood Cancer Treatment and Prevention

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Abstract

Background and Objective: Mesenchymal stem cells (MSCs) are multipotent stromal cells with the potential to differentiate into various cell types. Recently, the use of Wharton's jelly-derived mesenchymal stem cells (WJ-MSCs) has gained attention as a novel and accessible source for treating various diseases, including cancer, due to their numerous advantages. Furthermore, studies have demonstrated that the application of suitable scaffolds can significantly enhance the survival, proliferation, and differentiation of these cells. The present study aims to investigate the viability and adhesion of WJ-MSCs on a polylactic acid/wax (PLA/Wax) scaffold and its impact on the progression of acute myeloid leukemia (AML) and cancer treatment.

Materials and Methods: The PLA/Wax scaffold was fabricated in a 7:3 ratio using an electrospinning technique. The adhesion and viability of the isolated cells on these scaffolds were assessed through scanning electron microscopy (SEM) and the MTT assay. Complementary analyses, including immunofluorescence, Western blotting, and Fourier-transform infrared spectroscopy (FTIR), were conducted alongside histopathological studies of bone marrow to evaluate the efficacy and density of bone marrow transplantation (BMT) and to investigate the

therapeutic progression of leukemia.
Results: The results from SEM studies indicated that the fibers were homogeneous, uniform, free of beads, and of high quality. Additionally, the incorporation of wax into the PLA significantly reduced the fiber diameter. These findings confirmed that the cells adhered in large numbers and exhibited appropriate dispersion on the scaffold, representing a breakthrough in the field of high-quality bone marrow transplantation and leukemia treatment. Furthermore, the MTT assay demonstrated satisfactory biocompatibility of the fabricated scaffold with the cells, showing a significant increase in the survival rate of MSCs throughout the observation period.

Conclusion: the use of the PLA/Wax scaffold enhances cell adhesion, viability, and proliferation. This approach may hold significant potential in the treatment of hematological malignancies through bone marrow transplantation, reduction of tissue inflammation, and modulation of differentiation in defective hematopoietic stem cells. The present study introduces a novel therapeutic strategy for blood cancer, utilizing the described methodology to advance treatment options in this critical area of medicine.

Keywords: Mesenchymal stem cells (MSCs), Leukemia, Cancer Therapy, Cancer Prevention, PLA/Wax Scaffolds

1- Introduction

Mesenchymal stem cells (MSCs) are among the most potent types of stem cells. In recent years, advancements in tissue engineering have provided unprecedented opportunities for developing and refining therapeutic strategies to treat a variety of congenital and acquired diseases, including leukemia. Iran is the second country in the world, after Italy, in terms of the number of bone marrow transplantations (BMTs) performed. The incidence of adverse effects associated with BMT in Iran is lower compared to advanced centers in other countries [1]. Studies have demonstrated the efficacy of scaffold utilization in enhancing the differentiation, survival, and proliferation of stem cells. MSCs are multipotent stromal cells that exhibit a remarkable capacity for self-renewal and differentiation into a diverse array of cell lineages. These versatile stem cells possess strong myogenic potential and can be readily isolated from various tissue sources, including the umbilical cord, bone marrow, adipose tissue, dental pulp, and others. In controlled in vitro environments, MSCs can differentiate into mesodermal, ectodermal, muscular, neuronal, and endodermal cell types, including pancreatic islet cells and hepatocytes [2]. Notably, the most significant application of this property lies in the prevention or inhibition of cancer growth.

The umbilical cord and adipose tissue have been recognized as primary sources for the isolation and extraction of MSCs. Wharton's jelly of the umbilical cord contains connective

tissue derived from the extraembryonic mesoderm and fibroblast-like cells. MSCs isolated from Wharton's jelly present characteristics that are more primitive and intermediate between those of mature and embryonic stem cells. This unique developmental status of Wharton's jelly-derived Mesenchymal stem cells (WJ-MSCs) has garnered significant interest due to their potential applications in cancer prevention or as adjuvant therapy [3]. Importantly, the extraction of these cells is a painless procedure, and they possess a high proliferation rate. Additionally, the use of WJ-MSCs is ethically approved, as they do not form tumors [4], have low immunogenicity, and do not cause significant side effects in the recipient [5].

The integration of tissue engineering and cell therapy holds significant potential for the development of replacement tissues and the treatment of various diseases, particularly hematological malignancies such as leukemia. The primary aim of tissue engineering is to create replacement tissues and organs or to restore diseased or damaged parts in vivo. In recent years, the ability to generate controlled three-dimensional constructs for tissue engineering applications has advanced considerably [6]. To enhance cell yield under laboratory conditions, the use of three-dimensional cell culture systems can be beneficial, as these microenvironments more closely recapitulate the in vivo developmental conditions compared to traditional two-dimensional cultures. This approach facilitates the generation of larger and more physiologically relevant cell populations for therapeutic applications [7].

Nanofiber scaffolds characterized by their intricate network of interconnected fibers and numerous pores closely mimic the extracellular matrix and bone marrow stroma, thereby providing a favorable cellular microenvironment. This integration of cell therapy, tissue engineering, and scaffold development for cell attachment and growth holds significant promise for advancing treatment strategies for various diseases [8]. Since proper cell morphology is crucial for function and differentiation, scaffolds that closely mimic the native cellular microenvironment can promote optimal cell attachment, proliferation, and differentiation [9]. Various techniques have been proposed for scaffold fabrication, with electrospinning standing out as a prominent method. This technique facilitates the transformation of diverse natural and synthetic biodegradable polymers into nanofibers suitable for tissue engineering applications.
Studies have demonstrated that the use of Polycaprolactone (PCL) nanofiber scaffolds promotes the proliferation and differentiation of neuronal and muscle cells [10]. Additionally, evidence suggests that the use of electrospun scaffolds composed of nanofibers can serve as a geometric guide, enhancing the attachment and positioning of skeletal muscle cells. To this end, a combination of electrospinning and electrospray techniques was employed to fabricate an electroactive scaffold of polyurethane urea (PUU) nanoparticles, which successfully increased the proliferation and differentiation of C2C12 myoblast cells. In a study of neuronal cells, researchers observed that the PPY/PCL scaffold presented high biocompatibility and effectively promoted the differentiation of

PC12 cells. These findings indicate that nanofiber scaffolds hold promising potential for neural tissue engineering and enhance the adhesion and proliferation of these cells [11].

In other studies, the collagen/PPY fiber scaffold successfully enhanced the attachment of human bone marrow-derived MSCs and increased the expression of neural cell marker genes. In 2011, studies were conducted on the generation of definitive endoderm from embryonic stem cells on three-dimensional biodegradable PLCA scaffolds, and the results of this differentiation were compared to those in two-dimensional monolayer cultures. Gao and colleagues cultured embryonic stem cells on alginate or three-dimensional scaffolds and examined and compared their differentiation into definitive endoderm under both three-dimensional and two-dimensional conditions [12]. E Hoveizi and colleagues applied a new electrospun PLA/Wax/Cs scaffold for the differentiation of embryonic stem cells (ESCs) into neuronal cells and observed that the neuronal cells firmly attached and spread on the wax-containing scaffold. This represents an inspiring and promising concept in the field of leukemia as well. Additionally, the combination of natural materials with PLA resulted in uniform fibers with a smaller diameter [13]. In the present study, a combination of wax and PLA was used to fabricate a novel scaffold to enhance cell culture yield and attachment. To this end, PCL/Wax scaffolds were prepared by electrospinning, and the adhesion and survival of cultured MSCs on the scaffold were evaluated using various methods.

2- Methodology

Isolation and Culture of Mesenchymal Stem Cells (MSCs) from Wharton's Jelly

Umbilical cord blood was collected immediately after delivery and transported to the cell culture laboratory. The umbilical cord tissue was then washed with phosphate-buffered saline (PBS) containing antibiotics (penicillin, streptomycin) and amphotericin to remove any potential contaminants. Following thorough washing of the tissue, the stem cells were isolated using the explant method. Primary cells isolated after the first passage were initially stained for flow cytometry analysis with anti-CD90, anti-CD133, and anti-CD105 antibodies, which are markers of MSCs, as well as the anti-CD34 antibody, a marker of hematopoietic stem cells (HSCs). These cells tested negative for all of these markers. After confirmation of the cells' identity, they were transferred to culture flasks and incubated at 37°C, 5% CO2, and 59% humidity for 4 weeks. The culture medium was replaced every 3 days, and the cells were observed under a microscope daily [14].

Preparation of PLA/Wax Nanofiber Scaffold by Electrospinning

A PLA/Wax nanofiber scaffold with a 7:3 ratio was fabricated using the electrospinning technique. The scaffold was prepared with a PLA concentration of 2.5% and a wax concentration of 2.5%, dissolved in a solvent mixture of Hexafluoroisopropanol (HFIP) at a ratio of 9:4. The morphology, porosity, nanofiber size, and cell attachment to the scaffold

were evaluated using SEM, and the cytotoxicity of the scaffold was assessed using the MTT assay.

Culture of Mesenchymal Stem Cells on PLA/Wax Scaffolds

After preparation, the PLA/Wax scaffold was cut into 16 mm diameter pieces and sterilized by exposure to UV irradiation for 3 hours. The scaffold pieces were then placed in a 24-well plate and incubated in PBS containing high concentrations of penicillin (200 U/mL) and streptomycin (200 mg/mL) for 24 hours. Subsequently, 5 × 10^5 MSCs per well were seeded onto the scaffold.

Morphological Evaluation by Scanning Electron Microscopy (SEM)

The morphology, fiber diameter, and porosity of the prepared PLA/Wax scaffold, as well as the cellular arrangement on the scaffold, were examined using an FEI Quanta 30-XL SEM. To prepare the cell-laden scaffolds for SEM analysis, the samples were first washed with PBS for 10 minutes and fixed with 2.5% glutaraldehyde for 1 hour. Dehydration was then performed using a graded series of ethanol (30%, 50%, 70%, 80%, 90%, and 100%) for 15 minutes each. Next, the scaffolds were sputter-coated with gold particles and imaged using SEM.

Fiber diameter and pore size were measured using ImageJ software (version 10). For this purpose, five SEM images were selected, and from each image, 20 regions were randomly chosen to calculate the fiber diameter. The average of these measurements was then reported as the average fiber diameter [15].

Evaluation of Cell Proliferation and Viability by MTT Assay

To assess the survival and proliferation of the cells cultured on the scaffold, the MTT assay was performed at a concentration of 0.8 mg/mL. The MTT powder was dissolved in PBS at specified ratios and stored in the dark at 3°C until use. The assay was conducted on days 1, 3, 5, 7, and 9 after seeding the cells in the three-dimensional culture environment. For this assay, the cell culture medium was first removed, and 2 mL of fresh medium containing 200 µL of the MTT solution was added to each well. The plates were then incubated at 37°C for 3 to 4 hours. After incubation, 2 mL of dimethyl sulfoxide (DMSO) (Merck) was added to each well, and the absorbance was measured using an ELISA reader at a wavelength of 570 nm. To confirm the results, the experiment was repeated three times.

Evaluation of Cell Adhesion and Proliferation on the Scaffold

To evaluate cell adhesion and proliferation, MSCs were cultured on the PLA/Wax scaffolds in complete culture medium for 1, 3, 5, 7, and 9 days. Afterward, the cells were fixed with 4% paraformaldehyde and stained with DAPI (4',6-diamidino-2-phenylindole) at a concentration of 1 µg/mL. For cell counting, 20 random fields (each with an area of 1 mm^2) were selected each day, and the cells were counted. The average cell number per field was then calculated.

Western Blot Analysis

Western blotting is a laboratory technique used to detect specific protein molecules within a mixture of proteins. In the present study, this technique was employed to assess the levels of inflammatory proteins produced in the bone marrow tissue of both control and cancerous groups. In the control group, we observed the expression of phosphorylated CDK4 protein, with a band at 143 kDa. Conversely, in the WJ-MSC treatment group, due to a relatively modest initial reduction in inflammation, the inflammatory line of this protein was confirmed to be expressed in its initial form as CDK2, with a band at 65 kDa. Ultimately, in the WJ-MSC + PLA/Wax treatment group, a significant reduction in inflammation was observed, alongside the persistence of the Jak-2 line at 83 kDa. This finding supports the therapeutic potential of the treatment and confirms the proper engraftment of the administered cells within the bone marrow niches, thereby contributing to leukemia treatment and mitigating the body's immunological response.

Bone Marrow Transplantation: Evaluation of Definitive Treatment and Cancer Prevention

Bone Marrow Transplantation: Evaluation of Definitive Treatment and Cancer Prevention After obtaining the graft tissue using the aforementioned methods, the patient is administered a high dose of chemotherapy drugs (methotrexate, 200 mg/kg) along with radiation therapy to destroy the remaining bone marrow. In the final step, the healthy bone marrow is warmed and infused into the patient via a central venous catheter to replace the destroyed bone marrow. Once the graft tissue enters the bloodstream, the transplanted cells migrate to the bone marrow and begin producing new white blood cells, red blood cells, and platelets.

All stages of bone marrow transplantation were conducted precisely according to the proposed protocol by Hofbrand. The drug loading of methotrexate was performed using the "direct bone marrow introduction" mechanism, a conventional method in drug delivery for hematological malignancies. Notably, the bone marrow transplant achieved a success rate of 98.8%, as evidenced by the assessment of Sox9 signaling (Fig. 7-A) and HLA evaluations through immunofluorescence testing (Fig. 7-B) ($P < 0.0001$), which is considered an exceptionally favorable percentage in the field of bone marrow graft acceptance. Further details regarding the remaining concentrations will be provided in subsequent sections.

Data Analysis

The results were analyzed using GraphPad Prism software and subjected to statistical tests, including two-way ANOVA and t-tests, with data presented as mean ± standard error (SE). Graphs were generated using Microsoft Excel, and differences with $p < 0.05$ were considered statistically significant. All experiments were performed with at least three independent biological replicates.

3- Results

A. Findings:

Morphological Evaluation of Human Umbilical Cord Wharton's Jelly-Derived Mesenchymal Stem Cells (MSCs)

Seven days after placing Wharton's jelly tissue pieces in culture flasks containing culture medium, cellular outgrowths gradually began to emerge from the edges of the tissue. The cells were initially spherical in shape but became elongated and spindle-shaped over time (Figure 1A). Three days later (on day 10), the cells had covered the bottom of the flasks. Some impurities were observed in the first passage, but the cells appeared purer in subsequent passages. The MSCs adhered to the bottom of the flasks and fully covered the surface after a few days. As shown in Figure 1B, these cells exhibited the typical morphological characteristics of MSCs, with a spindle-shaped appearance and high adhesion and proliferation capacity in the early passages.

Morphological Evaluation of Scaffold and Cultured Mesenchymal Stem Cells (MSCs) on Scaffold

SEM was employed to evaluate the morphology and diameter of nanofibers in PLA/Wax scaffolds, as well as the arrangement of human umbilical cord WJ-MSCs on these scaffolds. SEM micrographs of the scaffolds are shown in Figure 2A. As observed in Figure 2, the nanofibers obtained from the blending of PLA with wax were uniform and free of beads. Figure 2B shows the arrangement of human umbilical cord WJ-MSCs on the PLA/Wax scaffold after 3 days, indicating high-density cell adhesion, spreading, and retention on the scaffold. The average fiber diameter was estimated to be 75 nm using measurement software (Figure 2).

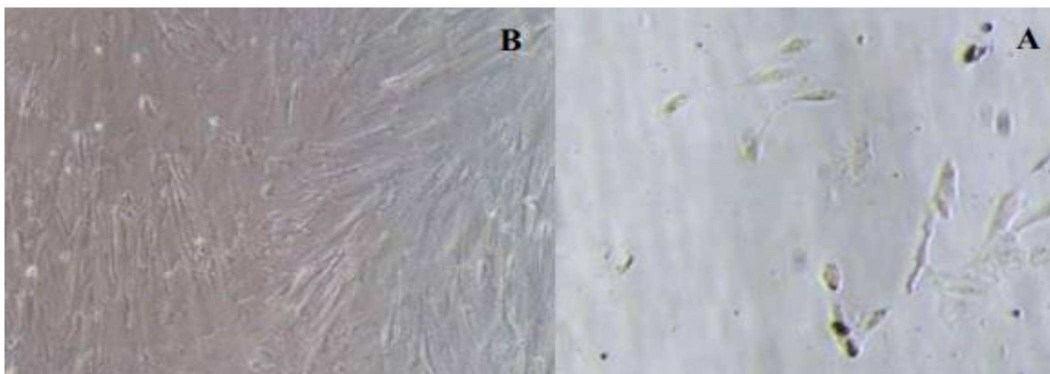

Fig 1. Morphological evaluation of mouse Wharton's jelly-derived mesenchymal stem cells (WJ-MSCs) using an inverted microscope. (A) Emergence of stem cells from Wharton's jelly on day 10 after seeding, (B) Complete coverage of the flask surface after detachment and proliferation of mesenchymal stem cells. Magnification 30X.

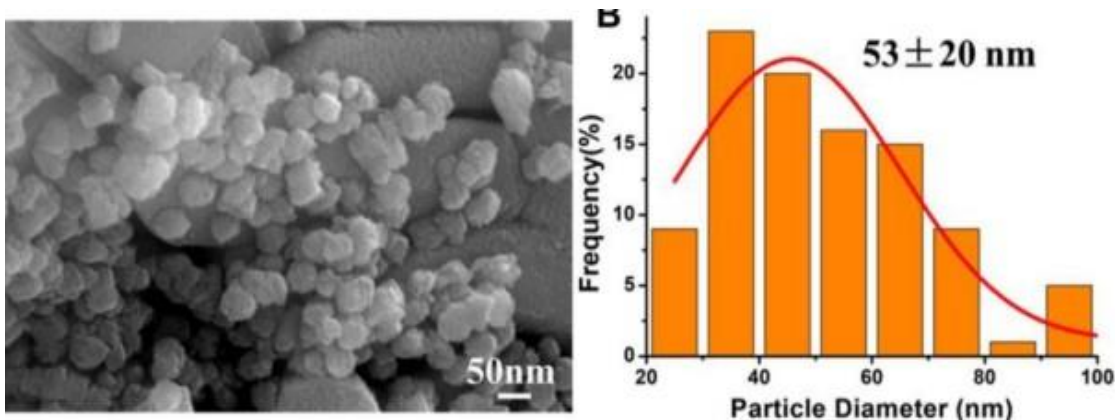

Fig 2. Morphological evaluation of scaffolds and human Wharton's jelly-derived mesenchymal stem cells (WJ-MSCs) cultured on the three-dimensional scaffold using SEM. (A) Uniform nanofibers of the electrospun PLA/Wax scaffold, (B) Graph showing the average fiber diameter of the nanofiber scaffold. The average diameter was estimated to be 53 nanometers(nm).

Evaluation of Cell Survival Using the MTT Assay

The MTT assay was performed to compare and evaluate the survival of human umbilical cord WJ-MSCs on the PLA/Wax scaffolds on days 1, 3, 5, and 7. The results indicated an increase in the survival of these cells on the scaffold over the 7-day period. The comparison of cell survival on this scaffold after one day did not show a significant difference from the two-dimensional culture sample used as a control. However, after 3 to 5 days of cell attachment on the scaffold, increased cell survival was observed, and after 7 days, cell survival on the scaffold was significantly higher (Figure 3).

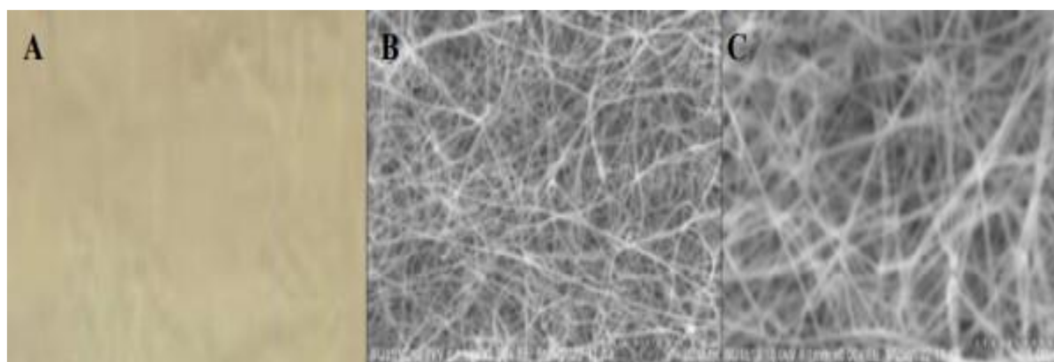

Fig 3. (A) Actual image, SEM image at (B) 2 K magnification (C) 6 K magnification of sample PLA/Wax

Evaluation of Cell Adhesion on the Scaffold

To assess cell adhesion, growth, and proliferation, MSCs were cultured on the PLA/Wax scaffolds using complete culture medium for 7 days. Observations indicated that the adhesion and proliferation of the MSCs on the scaffold were successful. As shown in Figure 4, the comparison of cell proliferation after 5 and 7 days revealed a highly significant difference in cell proliferation compared to day 1. These results demonstrate that the PLA/Wax nanofiber scaffold provides a suitable platform for MSCs attachment, growth, and proliferation (Figure 4).

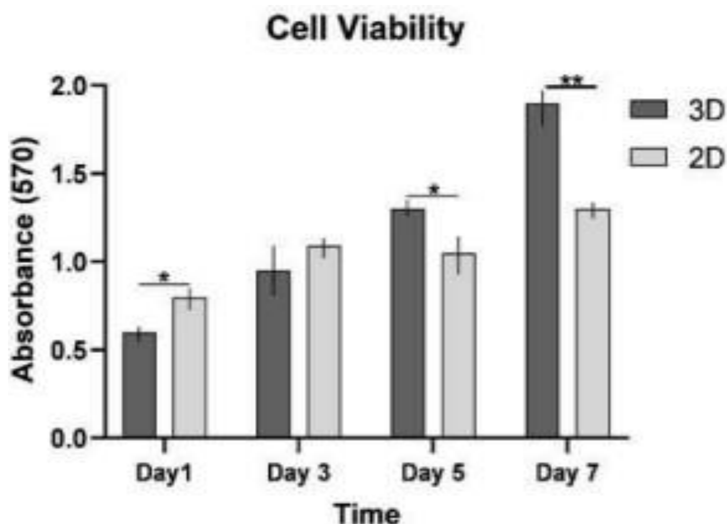

Fig 4. Graph showing the survival of human Wharton's jelly-derived mesenchymal stem cells (WJ-MSCs) on days 1, 3, 5, and 7 after culture, using the MTT assay. The values represent the mean of three experimental replicates. *p<0.05, **p<0.01, ***p<0.001 (D:2 is two- dimensional culture, and D:3 is three-dimensional culture).

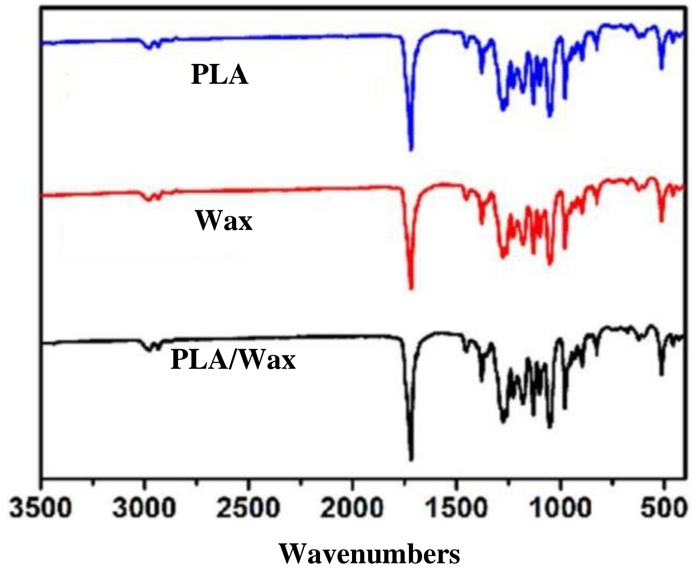

Fig 5. FTIR spectrum of PLA, Wax, and PLA/Wax nanofiber scaffolds.

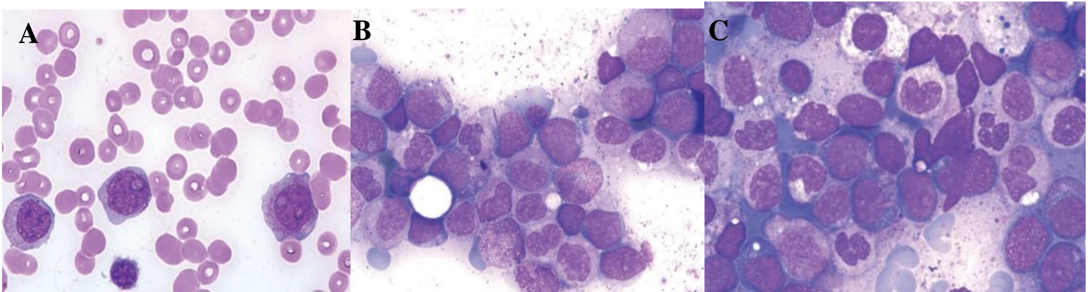

Fig 6. Histological images of Acute Myeloid Leukemia A) Peripheral blood smear. B, C) Bone marrow smear.

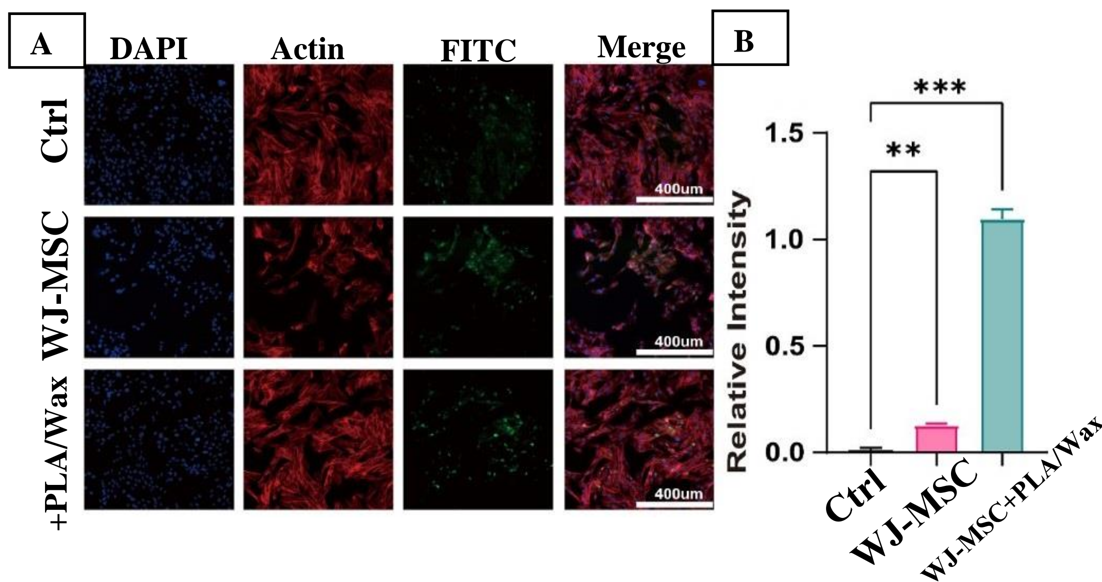

Fig 7. A) Effect of PLA/Wax nanofiber on WJ-MSC. Representative images of immunofluorescence of Sox9 of AML-BMSCs treated with different PLA/Wax nanofiber. B) immunofluorescence intensity analysis of Sox9 (B) of WJ-MSC treated with PLA/Wax nanofiber. Results were expressed as means ± SD; ns means no significant difference. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$.

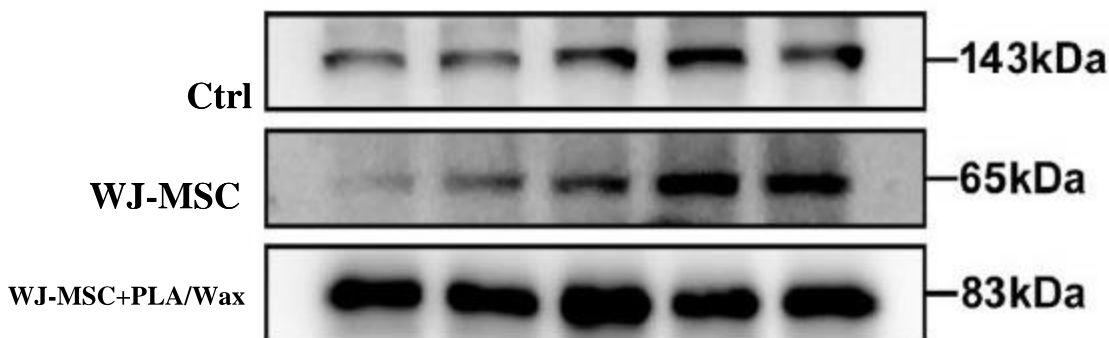

Fig 8. Effects of PLA/Wax scaffolds on Wharton's Jelly mesenchymal stem cells: Western blot results.

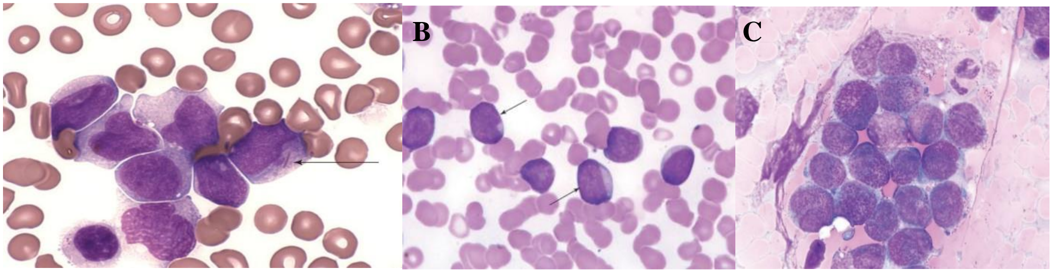

Fig 9. A, B) The effect of mesenchymal stem cells (MSCs) derived from Wharton's jelly on the bone marrow of a case with acute myeloid leukemia, which shows an increase in the efficiency and quality of bone marrow transplantation. C) Bone marrow smear.

4- Discussion and Conclusions

Stem Cell Therapy: A Promising Approach for Disease Treatment
Cell therapy has yielded remarkable successes in the treatment of various diseases, particularly those of hematological origin. To date, stem cells and progenitor cells from diverse sources have been employed in cell therapy, including embryonic stem cells, bone marrow stem cells, umbilical cord blood stem cells, and certain endoderm- and ectoderm-derived stem cells [1, 25]. In this study, a polylactic acid/wax (PLA/Wax) scaffold was fabricated, and the behavior, survival, and adhesion of cultured MSCs derived from Wharton's jelly on the scaffold, as well as their impact on the mouse bone marrow transplantation process, were investigated. WJ-MSCs were cultured on this three dimensional scaffold for seven days, and their adhesion and survival rates were evaluated as prerequisites for our novel bone marrow transplantation method.

SEM images of the electrospun three-dimensional PLA/Wax scaffold revealed uniform and smooth nanofibers of high quality, with no bead formation. To assess the biocompatibility of this scaffold, the MTT assay was performed on WJ-MSCs cultured on the scaffold over different time points. The results demonstrated that a significant number of cells adhered to the nanofibers and exhibited remarkable survival. Additionally, the images indicated the infiltration and presence of cells within the underlying layers of the scaffold.

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Numerous studies have highlighted the crucial role of three-dimensional scaffolds in facilitating the differentiation of stem cells into various cell types and in creating an environment more akin to the in vivo state, thereby enhancing their survival and proliferation. In the fields of genetic engineering and tissue engineering, synthetic polymers such as PLA hold significant importance and utility due to their biodegradability and biocompatibility [16]. Furthermore, these polymers can be used for the regeneration and repair of various tissues, including bone, skin, vascular tissue, and other organs, which is particularly relevant in the context of this study's focus on the repair of bone marrow tissue in leukemia patients [17,18].

Various studies have been conducted on different types of scaffolds, with the primary objective of developing biocompatible structures that effectively mimic the three-dimensional architecture of native body tissues. This allows for cell culture under conditions that closely mimic the natural environment, ultimately achieving optimal tissue repair.

In the present study, the addition of wax to the PLA scaffold aimed to reduce the thickness of the resulting fibers, improve the adhesion of cultured WJ-MSCs , and enhance their growth and proliferation through the electrospinning process. The findings of this study align with and support the results reported by Stenhamre and colleagues [19-23]. The results demonstrate that the PLA/Wax scaffold provides a suitable platform for the adhesion and growth of mouse umbilical cord WJ-MSCs. Furthermore, the findings confirm a significant increase in cell survival and proliferation when using the PLA/Wax scaffold.

Based on the secondary outcomes of bone marrow transplantation in mice with acute myeloid leukemia (AML), it can be concluded that this cell culture and therapy method represents a novel and promising approach for the treatment and prevention of cancer progression. We hope that in the near future, the ethical barriers and regulations surrounding human umbilical cord-derived stem cells will be addressed, enabling more effective treatment for patients with various types of leukemia.

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