

Repopulation of multipotent stem cells derived from adult male rabbits on a polycaprolactone scaffold: an invitro study

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ABSTRACT

Background: MSCs (mesenchymal stem cells) are multipotent, spindle-shaped cells that give them the ability to differentiate into a broad lineage of tissues. Polycaprolactone (PCL) is a synthetic material that has been used several times for the fabrication of scaffolds to be utilized in the field of tissue engineering.

Aim of the study: The main goal of this research is to develop a protocol for isolating, cultivating, and characterizing rabbit bone marrow mesenchymal stem cells (BMSCs), as well as to investigate their capacity to repopulate PCL scaffolds.

Materials and Methods: The bone marrow was harvested from femurs and tibias of 2 New Zealand rabbits. The harvested cells were cultured using complete culture medium. Cells from passage 3 were used for characterization of MSCs which done using flowcytometry. The bone marrow mesenchymal stem cells (BM-MSCs) were seeded over PLC scaffolds prepared with electrospinning. The PLC scaffold was evaluated by electron microscope before and after repopulation with cells.

Results: In Dulbecco's Modified Eagle's Medium (DMEM) supplied with 10% fetal bovine serum (FBS), BMSCs were successfully isolated, cultivated, and grown. The cells were characterized morphologically by their spindle-shape, processes of cytoplasmic, plastic adhesion tendencies, and formation of colonies. They were characterized by flowcytometric analysis as having a positive CD73 expression and a negative CD45 expression. Before cell seeding, the PCL scaffold was inspected under a scanning electron microscope to assess the PCL material characteristics and pour size, and after cell seeding to detect BMSC attachments to the scaffold.

Conclusion: The protocol used in this investigation was effective for isolation, culture, and characterization of rabbit BMSCs and the cells that were obtained successfully repopulated the PCL scaffold.

Keywords: Mesenchymal stem cells; Polycaprolactone scaffold; Flow cytometry; Cell seeding, Electron microscope.

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INTRODUCTION

In the realm of stem cells, (MSCs) are a prospective candidate for tissue engineering and cell-based treatment. MSCs are spindle-shaped plastic adherent cells generated from a number of host tissue, which include bone marrow¹ and other postnatal tissues.²⁻⁵ Despite the fact that MSCs are harvested effectively from a wide range of tissues, the most popular and accessible source is still bone marrow.⁶

Alexander Friedenstein⁷ discovered that MSCs might be isolated *in vitro* from hematopoietic cells in the

bone marrow. Arnold Caplan introduced the term "mesenchymal stem cells," hypothesizing that such cells may differentiate into a variety of mesenchymal tissues and proposing a wide lineage "tree" implying that MSCs can give rise to increased bone, cartilage, muscles, hematopoietic-supportive stroma, tendons, ligaments, adipose, as well as other connective tissue.⁸ Additionally, MSCs have the potential to produce a variety of biological substances that have paracrine regenerative and anti-inflammatory actions.⁹ Furthermore, they serve as a supporting stroma for hematopoietic stem cell (HSC) development and

differentiation and are thought to constitute one of their niches.¹⁰

MSCs are fibroblast-like fusiform cells. They form colonies in culture during their initial growth, which is known as a colony forming unit-fibroblast (CFU-F). Hematological surface markers such as CD34, CD45, and CD14 are negative, but Stro-1, CD44, CD29, CD105, CD73, CD166, and CD90 are positive.^{11, 12}

Polycaprolactone (PCL) is a biodegradable synthetic material with outstanding mechanical strength and durability, as well as biocompatibility. PCL is a water-soluble that works with the majority of 3D printing (3DP) systems available today. PCL's tailorable properties, which stem from its extremely low glass transition temperature and melting point, allow it to be used in 3DP devices.¹³ Prior research has shown that using PCL meshes as a scaffold for an artificial esophagus has promising outcomes. However, owing to the quick degradation of PCL, a pseudo-diverticulum formed around the mesh.¹⁴

The study aimed to assess the cell seeding capacity of rabbit BMSCs on PCL scaffolds for future tissue-engineered studies.

MATERIALS AND METHODS

All animal experiments and their care were carried out in accordance with the National Institute of Health's standards in the United States (NIH Pub. No. 85-23, revised in 1996). All animal studies were examined and authorized by the Faculty of Medicine at Al-Azhar University's Institutional Animal Care and Usage Committee (Approval Number Ped_1_11 Med. Research_Vivo generation /Esophageal Organoid _000011).

Rabbit BMSC isolation

The laboratory work was carried out at the tissue culture unit, Histology and Cell Biology Department, Ain Shams University. BMSCs were derived from two male New Zealand White rabbits that weighed about 2–2.5 kg and were cultured under complete aseptic conditions according to earlier published protocols.^{15, 16} Briefly, the rabbits were euthanized using the cervical dislocation method, and the femur and tibia of each rabbit were collected.¹⁷ To remove any attached soft tissue, the femur and tibia bones were gently scraped over sterile gauze. The epiphysis was cut with a medium-sized bone cutter, then Dulbecco's modified Eagle's medium (DMEM) (Lonza, Belgium) was used to flush out the bone marrow cavity; flushing was repeated till pale white bone became visible. To eliminate any bone spicules, soft tissues, or cell clumping, the cell suspension has been filtered using a 70-mm filtration mesh. Afterwards, the cell suspension was washed with phosphate buffer saline (PBS) (Invitrogen, UK) before being centrifuged at 2000 rpm for 5 minutes to obtain the final cell pellet. Trypan blue staining

was used to count the number of live cells, and the final cell pellets had been resuspended with 10 mL of full culture media before being cultured in the subsequent step.

Culture of rabbit BMSCs

The cells had been cultivated using complete culture medium (CCM), which included low-glucose DMEM mixed with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY) and 1% penicillin/streptomycin (Gibco). Non-adherent hematopoietic cells have been eliminated during the first cell passage once the colonies are noticeable after 10–14 days. The near confluent primary BMSCs were passaged using 0.25% trypsin/1mm ethylenediaminetetraacetic acid (EDTA) and re-plated at a density of 5×10^6 cells per 100 mm dish. The cells from the second and third passages were later employed in more studies. In a 37°C incubator containing 5% humidified CO₂, all cell cultures were kept in. Fresh CCM was added twice a week to the cell cultures.¹⁸

Characterization of rabbit BMSCs

For MSC cell surface marker expression, rabbit BMSCs (passage 3) were employed. As previously described, flow cytometry was used to evaluate the MSC surface markers, cluster of differentiation (CD) 73 and the hematopoietic markers CD45.^{19, 20} Using trypsin/EDTA, cells at passage 3 were collected. After cell counting, cells have been fixed for 30 min at a cell concentration of 3×10^6 cells/ml in a neutral 4% paraformaldehyde solution. The fixed cell suspension was rinsed twice with PBS before being stained with Fluorescein isothiocyanate (FITC) CD45 and CD73 monoclonal antibodies (Serotec, Kidlington, oxford, UK) and incubated for 30 min at room temperature in the dark. Cells have been pelleted, washed twice in PBS, and then fixed in 1 % paraformaldehyde in PBS for 15 minutes. A flow cytometer was utilized to identify cell surface antigens (Calibur, BD, USA).

Preparation of the PCL scaffold

Electrospinning PCL (Sigma Aldrich, UK) in an electrospay system was used to produce the PCL scaffolding (Nano NC, ESB-200, and South Korea). To achieve homogeneity, PCL powder was dissolved in glacial acetic acid to produce PCL solutions with a 20% W/V concentration. The resultant solution was mixed for 6 hours with a magnetic stirrer at (25–30°C) to produce a flat disc scaffold. A tiny metallic needle (diameter 0.7 mm) and a flat collector with a 20 kV voltage source were used to pump the solution at a rate of 1 ml/hour (Figure. 1). The scaffolds were all sterilized by being subjected to UV light for 24 hours.²¹

Rabbit BMSCs cell seeding on the fabricated PCL scaffolds *in vitro*

For 7-10 days, the PCL scaffolds were immersed in CCM containing rabbit BMSCs in an incubator at 37°C containing 5% humidified carbon dioxide. Three times a week, the CCM was changed. Under an electron microscope, the cellularized scaffolds were evaluated to determine the BMSCs' ability to adhere and repopulate the synthetic PCL scaffolds.



Fig 1: Polycaprolactone scaffold after fabrication

Scanning electron microscopic preparation of PCL before and after repopulation

Both types of scaffold (de- and re-cellularized) were kept in glutaraldehyde for scanning with an electron microscope. The specimens were dehydrated in a graduated ethanol series and dried at their critical point with a CP Dryer (Balzers, Lichtenstein). They were glued to aluminum stubs with carbon adhesive (Planocarbon, Groepl, Austria), then covered in a Baltech MED 020 coating system with a 10–15 nm gold-palladium layer. A Zeiss DSM 982 Gemini field emission scanning electron microscope with a voltage of 4–5 kV was used to examine the specimens. Photomicrographs at various magnifications were taken to get a clear view of the specimen's morphology.²¹

RESULTS

Bone marrow was extracted from male New-Zealand rabbits, and cells were seeded onto culture plates. BMSCs, which are bone marrow-derived adhering cells able to undergo substantial proliferation in primary culture, began to exhibit cytoplasmic processes with a fibroblastic appearance. Many cells tend to group together to form colonies. On the other hand, non-adherent hematopoietic contamination was expelled with the CCM change. Following 7 days of culture, the cell population was diverse; some were polygonal, whilst others were long, spindle-shaped, with a fibroblast-like appearance, and formed many colonies (Figure. 2a). During the second week, the number of adherent cells increased with noticeable colonies of opposing cells. The cultured cells were around 60% confluent on the 10th day, 70% to 80% confluent on the 15th day, and nearly 90% confluent on the 21st day of culture. Cells adopt various morphological characteristics, such as spindle, star, or sperm shape. Some of them were polygonal or

fibroblast like in appearance (Figure. 2b). After re-seeding, MSCs were polygonal, or spindle-shaped, with long processes during the first (Figure. 2c) and second (Figure. 2d) passages respectively, but fibroblast-like morphology began to prevail. At these points, they revealed a single phenotypic population. In terms of colony development and cellular morphology, all of the samples were similar. Higher magnification of the primary and first passages revealed granular cytoplasm, vesicular nuclei with many nucleoli indicating mitotic activity (3-4) (Figure.3).

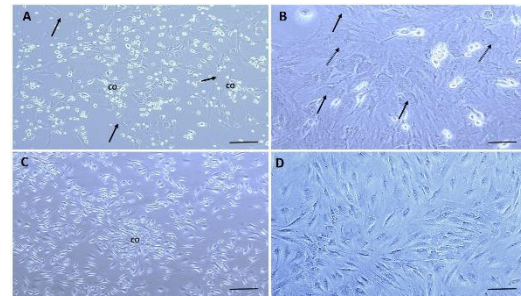


Fig 2: A phase contrast image of cultured rabbit BMSCs (A) after one week of cell culture, the cells had a polygonal shape similar to fibroblasts, with numerous cytoplasmic processes (arrow), and several colonies were visible (CO). Scale bar: 100µm. (B) after three weeks post culture, Scale bar: 20 µm. (C) BMSCs during the first passage. (D) BMSCs during the second passage.

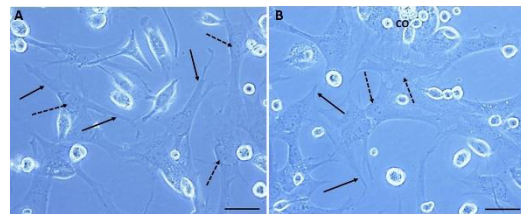


Fig 3: A phase contrast image of higher magnification of rabbit BMSCs (A) primary passage (B) first passage. Note: the cells exhibited granular cytoplasm (arrow) and abundant nuclei with multiple nucleoli (dashed arrow) and showed tendency to form colonies (CO).

MSCs were identified based on their morphological characteristics, plastic adherence, and colony formation. The majority of the cells were capable of colony formation that was sustained during the second passage. Flowcytometric analysis was done to validate cell identity using monoclonal antibodies against rabbit CD73 and CD45 to detect BMSCs using the surface markers CD73, and hematopoietic cell contamination was detected with the surface marker CD45. The MSC marker CD73 was expressed in the majority of the cells (91.5±3.2%). They were, on the other hand, negative for the hematopoietic marker CD45 (48.5±2.7%) (Figure. 4). As a result, bone marrow derived MSCs extracted using this technique were not contaminated with

hematopoietic cell lineages, with more than 91.5% being of mesenchymal lineages (Table 1).

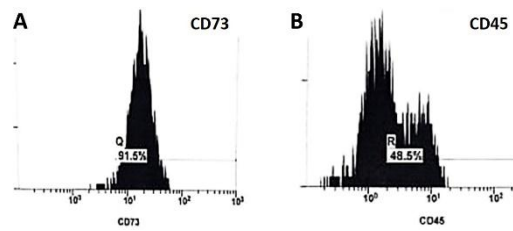


Fig 4: Flow cytometry analysis of BMSCs. (A) Most of the cells were expressed CD73 marker, while (B) showed the negative expression of the hematopoietic CD45 marker.

Stain	Percentage \pm SD
CD73	91.5 \pm 3.2%
CD45	48.5 \pm 2.7%

Table 1: Percentage of CD markers expression of MSCs and hematopoietic cells in the cultured rabbit BMSCs.

The different characteristics of the resultant PCL scaffold manufactured using an electrospinning technique were examined with scanning electron microscopy (SEM). The PCL scaffolds' nanofibrous morphologies, as observed by SEM, are shown in (Figure. 5a). It revealed a plethora of pores of various sizes. The fiber sizes were in the submicron range. Using SEM ultra-micrographs, the architecture of the scaffold's macro- and micro-pores was displayed with suitable interconnectivity. After studying the ultra-micrographs, the mean diameter of the micro-holes in the scaffold was determined to be 168.5 \pm 2.1 μ m. In the same context, the SEM ultra-micrograph (Figure. 5b) revealed that the BMSCs adhered well to the surface of the scaffold. When BMSCs were evaluated 7 days after seeding, they successfully adhered to the porous scaffold surface, characterized by fusiform or polygonal shape.

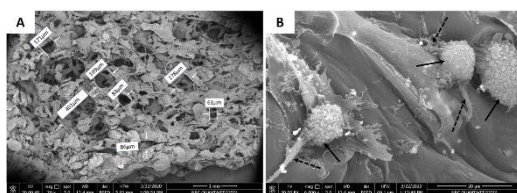


Fig 5: Scanning electron microscopy of the PCL scaffold before and after rabbit BMSCs seeding. (A) SEM of the PCL scaffold showing its highly porous structure with well-defined interconnected open pores, the average pore dimensions were 168.5 \pm 2.1 μ m. (B) Scanning electron microscopy of the PCL scaffold one week after seeding with rabbit BMSCs showing many attached cells with extracellular vesicles (arrow) and cytoplasmic processes (dashed arrow).

DISCUSSION

Tissue engineering is a novel treatment approach that involves the use of scaffolds, cells, and bioactive substances to rebuild damaged and diseased tissues. MSCs research is medically beneficial for less invasive cell treatment to accelerate tissue regeneration, decrease inflammation, and improve angiogenesis. The numerous techniques utilized to produce, characterize, and disseminate MSCs, however, complicate these studies, as well as the varied procedures for seeding these cells on various scaffolds.^{22, 23} The scaffold material criteria used in tissue engineering should be biocompatible with stem cells. Porous scaffolds promote cell penetration, adhesion, and proliferation.²⁴ In the present study, BMSCs harvested from rabbit bone marrow were successfully isolated, cultured and characterized using FACS. In addition, this study sheds light on the seeding capability of the isolated rabbit BMSCs on the PCL scaffold *in vitro*.

The primary requirements for a cell to be considered a MSC are its adhesion potential in monolayer culture under *in vitro* settings, as well as its ability to retain its undifferentiating properties over prolonged passaging.²⁵ This is similar with our findings in which rabbit BMSCs demonstrated adherent ability and were capable of significant proliferation in primary culture before exhibiting cytoplasmic processes with a fibroblastic appearance.

Consistent with prior research,^{26, 27} the cellular morphology of BMSCs in this study varied from polygonal to more spindle-shaped, with a fibroblast-like appearance. Furthermore, the capacity of BMSCs to form colonies, which is a basic indicator of progenitor cell activity. To verify the identity of cells and identify BMSCs, flow cytometry has been utilized. Flow cytometry revealed that cells exhibited positive MSC marker CD73 expression but negative for hematopoietic marker CD45, confirming the presence of BMSCs.^{19, 20, 26}

Tissue engineering is a growing science that allows for the development of functioning engineered tissues for regeneration without the drawbacks associated with conventional treatment modalities. To accomplish this objective, the scaffold should be permeable, biocompatible, and strong enough to promote cell development both *in vitro* and *in vivo*.²⁸ Recently, with the emerging nanofibrous technology, there has been a push to imitate the extracellular matrix and build scaffolds that are suitable for tissue engineering. These nanofibrous scaffolds are an attempt to mimic naturally occurring extracellular matrix components and may provide a better environment for tissue growth in tissue engineering systems.²⁹

Nanofibrous scaffolds composed of biodegradable polymer nanofibers may be created utilizing a variety of methods like electrospinning, self-assembling, and phase separation.³⁰ In the current investigation, electrospinning, a simple and effective technique, has been used to manufacture PCL, as described by Ihab,

*et al.*²¹ The resulting scaffold showed multi-pours with an average pore diameter of $168.5 \pm 2.1 \mu\text{m}$. After seeding the scaffold with rabbit BMSCs, a SEM revealed excellent cell adhesion to the scaffold's surface and pours. When cells were subjected to the scaffold, it had no degrading or inhibiting impact on cell growth. This came in agreement with previous reports with the Polycaprolactone scaffold with different types of stem cells.^{21, 31, 32}

CONCLUSION

Tissue engineering and regenerative medicine are bringing new promise to the restoration and repair of tissues and organs. The MSCs generated and cultured using the method described in this study demonstrated efficient indwelling on the Polycaprolactone scaffold, which may be built on in future research for maintaining a successful three-dimensional culture system for stem cells to be used for the purpose of tissue engineering of various organs.

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