Article

Discovery Medicine 2024; 36(181): 294–307
https://doi.org/10.24976/Discov.Med.202436181.28

Thermoresponsive and Injectable Pluronic F127 Hydrogel for Loading Adipose-Derived Mesenchymal Stem Cells

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Published: 20 February 2024

Background: Stem cell-based therapies display immense potential in regenerative medicine, highlighting the crucial significance of devising efficient delivery methods. This study centers on a pioneering approach that utilizes Pluronic F127 (PF127) as a thermoresponsive and injectable hydrogel designed for the encapsulation of adipose-derived mesenchymal stem cells (AdMSCs).
Methods: The degradation profile, gelation time, and microstructure of the PF127 hydrogel were thoroughly examined. AdMSCs were isolated, expanded, and characterized based on their multi-lineage differentiation potential. AdMSCs from the third passage were specifically employed for encapsulation within the PF127 hydrogel. Subsequently, the cytotoxicity of the AdMSC-loaded PF127 hydrogel was assessed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and apoptosis assays.
Results: Characterized by scanning electron microscopy (SEM), the PF127 hydrogel exhibited a porous structure, indicating its suitability for accommodating AdMSCs and facilitating wound healing. The PF127 hydrogel demonstrated reversible phase transitions, rendering it suitable for *in vivo* applications. Studies on the gelation time of PF127 hydrogel unveiled a concentration-dependent decrease in gelation time, offering adaptability for diverse medical applications. Analysis of the degradation profile showcased a seven-day degradation period, leading to the decision for weekly topical applications. Cytotoxicity assessments confirmed that AdMSCs loaded into the PF127 hydrogel maintained heightened metabolic activity for up to one week, affirming the safety and appropriateness of the PF127 hydrogel for encapsulating cellular therapeutics. Furthermore, cell apoptosis assays consistently indicated low rates of apoptosis, emphasizing the viability and robust health of AdMSCs when delivered within the hydrogel.
Conclusions: These findings underscore the vast potential of PF127 hydrogel as a versatile and biocompatible delivery system for AdMSCs in the realm of regenerative medicine. Boasting adjustable gelation properties and a remarkable capacity for cell encapsulation, this pioneering delivery system presents a promising path for applications in tissue engineering and wound healing. Ultimately, these advancements propel and elevate the landscape of regenerative medicine.

Keywords: Pluronic F127; hydrogel; adipose-derived mesenchymal stem cells; stem cell delivery; regenerative medicine; thermoresponsive; cytotoxicity; gelation time

Introduction

Hydrogels represent cross-linked polymer networks characterized by a higher water content, making them conducive to fostering cell viability [1,2]. Their elevated water content serves to hydrate the surrounding tissue and sustain a moist environment [3]. Moreover, hydrogels possess the ability to absorb tissue fluid, known as exudate, and expand until they reach equilibrium [3]. Additionally, these hydrogels are adept at serving as a platform for seeding stem cells, effectively emulating a three-dimensional cellular microenvironment that is crucial for ensuring optimal cell survival [4].

Pluronic F127, also recognized as Poloxamer 407 or PF127, stands as a hydrophilic thermoreversible hydrogel system extensively employed for drug delivery across various administration routes [5,6]. This polymer embodies thermosensitive, biocompatible, and bioabsorbable qualities, rendering it highly promising for applications in tissue engineering [7]. One of PF127's notable features lies

in its thermoreversible properties: assuming a fluid state at lower temperatures for ease of administration, while transitioning into a gel state at elevated temperatures, facilitating sustained release of encapsulated agents [8]. Consequently, PF127 at concentrations ranging from 18% to 50% solidifies into a hydrogel above 10 °C and reverts to a liquid state when cooled below this threshold [9].

Presently, PF127 finds utility in delivering mesenchymal stem cells (MSCs) to defect sites, demonstrating considerable potential in MSC-mediated tissue regeneration [10]. It furnishes a three-dimensional environment conducive to the differentiation of stem cells [11]. Employing PF127 hydrogel for seeding stem cells enhances cell survival at wound sites, thereby amplifying the effectiveness of cell-based therapy [4].

When adipose-derived stromal vascular fraction (AdSVF) cells are cultured, a specific subset of adherent cell populations is isolated, known as adipose-derived mesenchymal stem cells (AdMSCs). These AdMSCs exhibit therapeutic potential that contributes significantly to enhancing wound healing through various mechanisms. They facilitate the up-regulation of macrophage chemotaxis, promote tubulogenesis of endothelial cells by regulating cell adhesion, and stimulate fibroblast migration and angiogenesis [12,13]. Notably, the direct application of AdMSCs onto dermal wounds is associated with heightened granulation tissue formation and improves wound healing outcomes [14]. Furthermore, this approach has demonstrated superior efficacy compared to therapy involving bone marrow-derived mesenchymal stem cells (BM-MSCs) [14]. It's essential to highlight that the application of AdMSCs in conjunction with the extracellular matrix or when seeded in scaffolds shows more favorable outcomes in wound healing compared to utilizing AdMSCs alone [12].

Stem cells can be seeded in PF127 hydrogel to improve cell survival at the wound site and enhance the efficacy of cell-based therapy [4]. Hence, the current study sought to assess the capacity of a thermoresponsive and injectable PF127 hydrogel in encapsulating rabbit AdMSCs. Stem cells could be incorporated into the developed hydrogel preparations to augment cell survival at the wound site, thereby elevating the effectiveness of cell-based therapy. This investigation aimed to explore the potential of these stem cell-loaded hydrogel formulations as a topical therapeutic approach for improving wound healing.

Materials and Methods

Experimental Animals

Two healthy adult male New Zealand White rabbits, aged between 1 and 2 years (n = 2), were selected for the collection of interscapular adipose tissue. Before the commencement of the study, a two-week acclimatization period was observed for the rabbits, during which they were housed under standardized environmental conditions, maintaining a 12-hour light/dark cycle, and constant temperature and humidity levels. Additionally, the rabbits had continuous access to a standard diet and *ad libitum* drinking water [15].

Isolation, Expansion, and Characterization of Adipose-Derived Mesenchymal Stem Cells

The rabbits were anesthetized by the intramuscular injection of xylazine (Xylaxin, Catalog # 21, Indian Immunologicals Ltd., Hyderabad, Telangana, India) at 6 mg/kg body weight followed by ketamine (Aneket, Neon Laboratories Ltd., Thane, Mumbai, India) at 60 mg/kg body weight [16]. Subsequent to the surgical collection of interscapular adipose tissue, euthanasia was performed on the rabbits using an intraperitoneal injection of sodium thiopental, administered at an overdose level of 100 mg/kg body weight. The adipose-derived stromal vascular fraction (AdSVF) was prepared from the interscapular adipose tissue following a previously established protocol utilized in our laboratory [16]. Subsequently, the AdSVF cells were incubated in a complete culture medium, allowing adherence to cell culture flasks. These adherent cells were cultured in Dulbecco's Modified Eagle's Medium (Catalog # 11885084, Gibco, Carlsbad, CA, USA) supplemented with 15% fetal bovine serum (Catalog # 16140071, Gibco, Carlsbad, CA, USA) and 1% penicillin-streptomycin (Catalog # 15070063, Gibco, Carlsbad, CA, USA) solution, maintaining a controlled environment at 37 °C in a humidified atmosphere comprising 95% air and 5% CO_2. The culture medium was refreshed every 72 hours until the cells reached approximately 80% confluence. Upon reaching this confluence, the cells underwent trypsinization using a 0.25% trypsin-EDTA solution (Catalog # 25200056, Gibco, Carlsbad, CA, USA). Subsequent passages were conducted to expand the cell population, maintaining consistent culture conditions. For further experimentation, AdMSCs from the third passage (P3) were utilized. It's important to note that prior to use, the AdMSCs underwent testing for mycoplasma contamination using polymerase chain reaction (PCR).

To visualize the morphology of AdMSCs at the third passage (P3), crystal violet staining was performed. The adherent AdMSCs were gently rinsed with phosphate-buffered saline (PBS) (Catalog # AM9624, Invitrogen, Waltham, MA, USA) to eliminate residual medium and debris. Following this, the cells were fixed using 4% paraformaldehyde for 15 minutes at room temperature. A staining solution containing crystal violet (Catalog # C0775, Sigma-Aldrich, St. Louis, MO, USA) was added and allowed to incubate for 10 minutes before rinsing off the excess stain with PBS. The stained AdMSCs were observed and analyzed under a light microscope (Olympus CKX53, Shinjuku-ku, Tokyo, Japan).

Trilineage Differentiation Potential of Adipose-Derived Mesenchymal Stem Cells

The trilineage differentiation potential of the expanded AdMSCs towards adipogenic, chondrogenic, and osteogenic lineages was assessed following the third passage, employing specific differentiation kits as per the manufacturer's instructions [17]. AdMSCs were seeded at a density of 1×10^4 cells/cm^2 in a 24-well plate. Control plates were maintained with a standard culture medium, which was replaced every three days.

Osteogenic differentiation: The cells were initially incubated in a complete culture medium for four days within a CO_2 incubator. Following this incubation period, the medium was replaced with a pre-warmed complete osteogenesis differentiation medium (Catalog # A1007201, Gibco StemPro, Carlsbad, CA, USA), and the cells were further incubated for 24 days. The osteogenesis differentiation medium was renewed every three days throughout this duration. After the 24-day incubation, the medium was removed, and the cells were gently washed with PBS. Subsequently, fixation was performed using 4% formaldehyde for 30 minutes. The wells were thoroughly rinsed with PBS and then stained with a 2% Alizarin Red S solution (Catalog # A5533, pH 4.2, Sigma-Aldrich, St. Louis, MO, USA) for 10 minutes. Following staining, the plates were washed with PBS, and the stained AdMSCs were observed under a light microscope.

Adipogenic differentiation: The cells were initially incubated in a complete culture medium for two days within a CO_2 incubator. Subsequent to this initial phase, the medium was replaced with a pre-warmed complete adipogenesis differentiation medium (Catalog # A1007001, Gibco StemPro, Carlsbad, CA, USA), and the cells were further incubated for a period of 28 days. The adipogenesis differentiation medium was refreshed every three days during this timeframe. After the 28-day incubation, the medium was removed, and the cells were gently rinsed with PBS. Following rinsing, fixation was carried out using 4% formaldehyde for 30 minutes. The wells were washed with PBS, and subsequently, 60% isopropanol was added and discarded after a five-minute incubation. Thereafter, the cells were stained with Oil Red O stain (Catalog # O0625, Sigma-Aldrich, St. Louis, MO, USA) working solution for 20 minutes. Following staining, the plates were washed with PBS, and the stained AdMSCs were observed under a light microscope.

Chondrogenic differentiation: The cells underwent an initial two-hour incubation in a complete culture medium within a CO_2 incubator. Subsequent to this phase, the medium was replaced with a pre-warmed complete chondrogenesis differentiation medium (Catalog # A1007101, Gibco StemPro, Carlsbad, CA, USA), and the cells were further incubated for an additional 14 days. The chondrogenesis differentiation medium was refreshed every three days during this period. Upon completion of the 14-day incubation, the medium was removed, and the cells were gently rinsed with PBS. Fixation was performed using 4% formaldehyde for 30 minutes. Following fixation, wells were thoroughly washed with PBS and subsequently stained with a 1% Alcian Blue solution (Catalog # B8438, Sigma-Aldrich, St. Louis, MO, USA) for 30 minutes. The wells were then rinsed three times with 0.1N HCl. Following this, the plates were washed with PBS, and the stained AdMSCs were observed under a light microscope.

Preparation of Pluronic F127 Hydrogel

A solution of Pluronic F127 ($PEO_{100}PPO_{65}PEO_{100}$, Molecular Weight = 12.5 kDa) hydrogel at a concentration of 25% (w/v) was meticulously prepared by dissolving 2.5 g of PF127 powder (Pluronic® F127, Sigma-Aldrich, St. Louis, MO, USA) in 10 mL of ice-cold medium (Dulbecco's Modified Eagle's Medium containing 1% penicillin-streptomycin solution) [7,18]. The PF127 powder was gradually added to the centrifugation tube containing the ice-cold medium, which was maintained at a temperature between 4–10 °C, over a span of 2 to 3 minutes while gently mixing it. This gradual addition method effectively hydrated each flake, accelerating the dissolution process. Conversely, the rapid addition of PF127 to water results in a substantial clump formation that takes several hours to dissolve. Subsequently, the solution was stored at 4 °C overnight to ensure complete dissolution of the PF127 powder. The prepared solution underwent filtration using a 0.22 µm pore-sized syringe filter and was then stored at 4 °C until further use.

Scanning Electron Microscopy

Scanning electron microscopy (SEM) was employed to characterize the morphology and structure of the 25% PF127 hydrogel. The solid hydrogel maintained at 37 °C was subjected to a 24-hour freeze-drying process to eliminate the water content. The resulting freeze-dried specimens were affixed onto metallic stubs using double-sided carbon tape to ensure secure attachment. Prior to visualization under the SEM (Cube II, EMCrafts, Gwangju-si, Gyeonggi-do, South Korea), a thin conductive coating of gold was applied through sputter-coating (10 mA, 90 s, 2.0 $\times 10^{-2}$ torr) using the G20 Ion Sputter Coater (GSEM, Suwon-si, Gyeonggi-do, South Korea). This coating procedure was carried out to enhance conductivity before examining the hydrogel's structure and morphology.

Gelation Time in Physiological Conditions

Different concentrations of PF127 hydrogel were prepared using DMEM containing antibiotics (20%, 25%, 30%, and 40%). The mixture was refrigerated at 4 °C overnight for 12 hours to ensure complete dissolution. Subsequently, the final preparation underwent filter sterilization using a 0.22-µm pore-size syringe filter. Gelation time was determined following the protocol outlined by

García-Couce *et al.* (2022) [19]. For this evaluation, 2 mL of the prepared hydrogel solutions were transferred into glass vials to facilitate observation and stored at 4 °C. A temperature-controlled water bath was used to maintain the sample container at 37 °C. The timer was initiated immediately after placing the vial containing the PF127 solution into the temperature-controlled water bath. Observations were recorded and categorized as either "flow liquid sol" or "no flow solid gel" [20]. The vial was gently tilted at a slight angle, typically around 45 degrees, at regular 5-second intervals, while closely observing the solution's flow behavior.

The gelation time, signifying the transition of the PF127 solution into a gel state, was identified as the point when the solution ceased flowing or displayed resistance to tilting. Once an approximate time range (spanning 10 seconds) was determined, the experiment was repeated without lifting the vial at 5-second intervals (n = 5). For instance, if the initial time range fell between 80 and 90 seconds, the vial was continuously maintained at 37 °C for 80 seconds (n = 5) before tilting to observe gelation. This process was replicated at 85 (n = 5) and 90 seconds (n = 5). To determine the precise gelation time for each concentration, the readings were only recorded in the case of complete gelation was observed while tilting. Subsequently, the average value derived from these observations (maximum n = 15) was considered the gelation time for that particular concentration.

Degradation Profile of PF127 Hydrogel

The degradation profile of the PF127 hydrogel was assessed following the methodologies proposed by Diniz *et al.* [10] (2015) and Gao *et al.* [21] (2009). Initially, 500 µL of PF127 was incubated in a 2 mL Eppendorf tube within an incubator maintained at 37 °C and 5% CO_2 until gelation occurred. Subsequently, 1 mL of complete culture medium was carefully layered on top of the hydrogel and incubated for various time intervals (1, 3, 5, and 7 days). At each specified time point, the samples underwent centrifugation at 4000 ×g for 10 minutes at room temperature. Following centrifugation, the complete medium was meticulously removed for further analysis or characterization purposes. After the samples were recovered, the extent of weight loss was measured after carefully drying and weighing the specimens. The percentage weight loss at each time interval (n = 5) was calculated based on the equation given below.

$$\textbf{Weight loss}\ (\%) = \frac{W1 - W2}{W1} \times 100$$

W1: weight of the gel before degradation.
W2: weight of the gel after degradation.

Cytotoxicity Evaluation Using MTT Assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Catalog # M6494, Invitrogen, Waltham, MA, USA) was conducted following the protocol outlined by Tada *et al.* (1986) [22] with certain modifications. AdMSCs at passage 3 (P3), suspended in a complete medium, were added into 96-well plates after adjusting the cell density to 1×10^4 cells/well. After an initial 24-hour incubation at 37 °C and 5% CO_2, the culture medium was replaced. To prevent premature gelation, the PF127 hydrogel (25%) was kept on ice. The cells were divided into two groups based on the replacement culture medium: the experimental group (n = 3) received 100 µL of PF127 hydrogel (sol phase) made using DMEM, while the control group (n = 3) received 100 µL of complete medium. The plate was then placed at 37 °C and 5% CO_2 for 5 minutes to initiate gel formation. Subsequently, 100 µL of complete medium was overlaid onto the experimental group wells to prevent desiccation, and the plate was transferred to the incubator. The cells were cultured for 1, 3, and 7 days under these conditions.

Following the incubation period, the medium was carefully aspirated after being maintained at 4 °C for 15 minutes. The MTT reagent was prepared by dissolving MTT in sterile PBS to achieve a suitable concentration (5 mg/mL). A volume of 20 µL of the MTT reagent was added to each well, ensuring that the hydrogel was fully submerged. Subsequently, the plate was incubated at 37 °C and 5% CO_2 for a duration of 4 hours. After the incubation period, the MTT reagent was removed following centrifugation at 2000 RPM (REMI Laboratory Centrifuge R-8M Plus, REMI, Mumbai. Maharashtra, India) for 10 minutes. The wells were then gently washed to eliminate excess MTT. Subsequently, 100 µL of a 10% SDS solution (Catalog # 428023, Millipore, Burlington, MA, USA) was added to each well and incubated at 37 °C to dissolve the formed crystals. The plate was gently agitated to ensure complete dissolution of the formazan crystals and further incubated at 37 °C for 10–15 minutes. The absorbance of the solution in each well was measured using a microplate reader (Multiskan FC, Thermo Fisher Scientific Inc., Waltham, MA, USA) at a wavelength of approximately 570 nm. These measurements were conducted in triplicate (n = 3) to ensure the reliability and consistency of the results.

Cell Apoptosis Assay

The cell apoptosis assay utilizing 4′,6-diamidino-2-phenylindole (DAPI) stain (Catalog # D9542, Sigma-Aldrich, St. Louis, MO, USA) was conducted to detect and quantify apoptosis, following a protocol described by [23]. AdMSCs at passage 3 (P3), suspended in a complete medium, were added into 48-well plates after adjusting the cell density to 1×10^4 cells/well. After an initial 24-hour incubation at 37 °C and 5% CO_2, the culture medium was replaced.

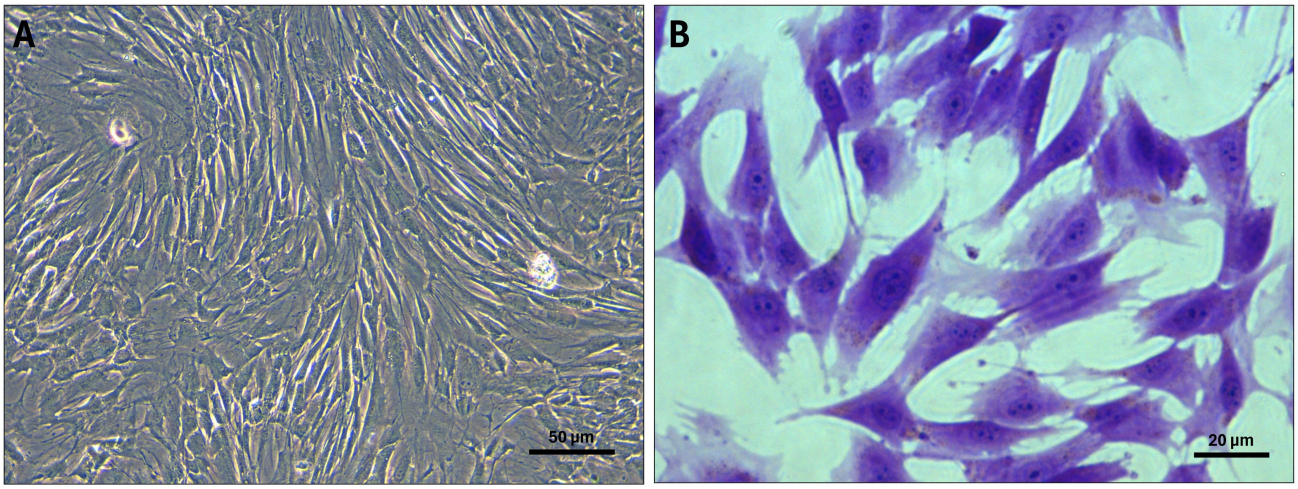

Fig. 1. Microscopic characteristics of adipose-derived mesenchymal stem cells (AdMSCs). (A) The third passage (P3) stage AdMSCs under a bright field microscope showing 80% confluence. (B) Crystal violet staining of P3 stage AdMSCs to visualize and assess the characteristics of stem cells.

To prevent premature gelation, the PF127 hydrogel (25%) was kept on ice. The cells were then divided into two groups based on the replacement culture medium: the experimental group was substituted with 200 µL of PF127 hydrogel (sol phase) prepared using DMEM, while the control group was replaced with 200 µL of complete medium. The plate was incubated at 37 °C and 5% CO_2 for 5 minutes to induce gel formation. Subsequently, 200 µL of complete medium was overlaid onto the experimental group wells to prevent desiccation, and the plate was transferred to an incubator, and further cultured for 3 and 7 days. After incubation, the media was carefully aspirated following a 15-minute incubation at 4 °C. The cells were then fixed using 4% paraformaldehyde for approximately 15–30 minutes to maintain their structural integrity. Post-fixation, the fixative solution was removed, and the cells were gently washed with PBS. Following this, a permeabilization buffer consisting of 0.1% Triton X-100 in PBS was added to each well. DAPI stain was applied to each well, ensuring coverage of the entire cell population, and the cells were incubated with DAPI for 10 minutes at room temperature under dark conditions. Under fluorescence microscopy (IX 71, Olympus, Shinjuku-ku, Tokyo, Japan), the results were interpreted based on nuclear morphology:

Normal cells (Non-apoptotic): In normal, healthy cells, the nucleus typically exhibits a uniform appearance, characterized by a diffuse blue staining pattern observed throughout the nucleus.

Apoptotic cells: Apoptotic cells often display condensed or fragmented nuclei, presenting as bright, punctate, or irregularly shaped structures. This distinct fragmented or condensed appearance within the nuclei serves as a characteristic indicator of apoptosis.

The percentage of apoptotic cells was quantified (manual counting) by analyzing the number of cells with apoptotic nuclear morphology relative to the total number of cells in the field of view (n = 6). The apoptosis rate (%) was calculated using the following formula:

$$\textbf{Apoptosis Rate}\ (\%) = \frac{\text{Number of apoptotic cells (in 100)}}{\text{Total number of cells (100)}} \times 100$$

Statistical Analysis

All data were presented as mean ± standard deviation (SD) of the mean. To evaluate differences between distinct groups, unpaired *t*-tests were employed. A significance level of $p < 0.05$ (*) was deemed statistically significant for all analyses.

Results

Cultural Characteristics of AdMSCs

Rabbit AdMSCs, similar to MSCs from other species, demonstrate a robust affinity for plastic surfaces. Upon seeding onto tissue culture plates, they promptly adhere to the surface, creating a monolayer of cells—an inherent trait defining MSCs. In culture, rabbit AdMSCs typically exhibit a spindle-shaped or fibroblast-like morphology, featuring elongated, slender cytoplasmic extensions surrounding a central nucleus. This particular morphology is a hallmark feature of MSCs. Their densely packed arrangement signifies a robust proliferation capacity. The firm adhesion of Rabbit AdMSCs to the plastic culture surface stands out as a distinctive trait of mesenchymal stem cells. They firmly anchor themselves to the substrate, maintaining robust attachment during media changes and handling procedures. These cells display the ability to undergo multiple passages while retaining their spindle-like morphology and strong adherence characteristics. Crystal violet staining provided visual evidence of their robust attachment, show-

casing a well-established monolayer formation on the substrate (Fig. 1). AdMSCs were observed to be firmly attached, forming a cohesive monolayer on the culture vessel.

Trilineage Differentiation

Trilineage differentiation represents a key characteristic of mesenchymal stem cells, involving their capability to differentiate into three specific cell lineages: osteogenic (bone), adipogenic (fat), and chondrogenic (cartilage) lineages. Subsequent to differentiation and staining procedures, the following observations were made.

Osteogenic Differentiation: Mesenchymal stem cells exhibit significant morphological alterations following successful osteogenic differentiation. They transit from a spindle-shaped, fibroblast-like appearance to a more cuboidal or polygonal shape, resembling osteoblasts—the cells responsible for bone formation. A distinctive feature of osteogenic differentiation involves the deposition of calcium salts within the extracellular matrix. This mineralization process can be visualized through Alizarin Red S staining. Upon staining with Alizarin Red S, the presence of calcium deposits within the differentiated cells and their surrounding matrix imparts a characteristic reddish-orange coloration (Fig. 2A,B). This staining pattern serves as confirmation of successful differentiation into the osteogenic lineage.

Adipogenic differentiation: Notable morphological changes prompt in stem cells as they accumulate lipid droplets. This process involves a shift from a fibroblast-like appearance to a round or oval shape, marked by visible intracellular lipid vacuoles. Staining with Oil Red O serves as a confirmation of adipogenic differentiation. This particular stain binds to lipid droplets, rendering them visible as red droplets within the cytoplasm (Fig. 2C,D). The intensity and abundance of red staining directly correlate with the extent of adipogenic differentiation. The presence of intracellular lipid droplets signifies a characteristic feature of adipogenic differentiation, indicating the transformation of these cells into adipocytes—cells primarily responsible for storing fat.

Chondrogenic differentiation: The typical formation of three-dimensional cell aggregates known as spheroids, representing the early stages of cartilage development. The assessment of this process often involves the utilization of Alcian Blue staining to identify glycosaminoglycans (GAGs), a primary component of the cartilage matrix. Upon staining, GAGs manifest as blue-colored entities. Successful chondrogenic differentiation leads to the accumulation of GAGs within these cell aggregates. The extent and intensity of the blue staining signify the degree of chondrogenic differentiation (Fig. 2E,F). These distinct visual markers serve as tangible confirmation of successful differentiation, playing a crucial role in evaluating the potential for stem cell differentiation.

Scanning Electron Microscopy

SEM images of the freeze-dried PF127 hydrogel revealed channel formations on the surface, characteristic of a network morphology (Fig. 3). Notably, pores were also evident across the surface. Overall, the hydrogel surface exhibited interconnected channels interspersed with pores, resulting in an irregular texture. These specific morphological attributes make it particularly suitable for loading AdMSCs. Moreover, the porous and loose network structure of PF127 hydrogel indicates its potential for promoting breathability, which can play a facilitative role in skin wound healing processes.

Physical Characterization of Pluronic F127 Hydrogel

The thermoreversible PF127 hydrogel underwent preparation and characterization following standard procedures. Macroscopic evaluation was conducted at two distinct temperatures: 4 °C (storage temperature) and 37 °C (physiological temperature). At 4 °C, the hydrogel presented as a liquid, while at 37 °C, it transitioned into a gel state (Fig. 4A). Notably, the gel formation observed was reversible, allowing the gel to return to a liquid phase by refrigeration. This reversible thermal behavior was found to be repeatable innumerably without causing alterations to the physical properties of the hydrogel.

Gelation Time

The gelation times of PF127 hydrogels at varying concentrations (20%, 25%, 30%, and 40% PF127) were assessed and plotted (Fig. 4B). The findings illustrate an inverse relationship between gelation time and PF127 concentration. Specifically, there is a notable decrease in gelation time with the increase in PF127 concentration from 20% to 40%. Such concentration-dependent behavior is characteristic of thermosensitive hydrogels like PF127.

Degradation Profile of PF127

The analysis of the hydrogel degradation profile data revealed rapid degradation of the PF127 hydrogel over the span of one week (Fig. 4C). Within this timeframe, the hydrogel exhibited almost a 50% reduction in its total weight when incubated in a regular culture medium. It is important to note that when applied to the wound surface, the degradation rate is expected to be significantly higher. As a result, a weekly application of the hydrogel topically would be optimal to ensure sustained release and effectiveness.

Cytotoxicity of PF127

The cytotoxicity and metabolic activity of AdMSCs loaded within PF127 hydrogel were assessed using the MTT assay on days 1, 3, and 7. Notably, the AdMSCs loaded in the hydrogel displayed sustained high metabolic activity for up to one week of incubation, affirming the suit-

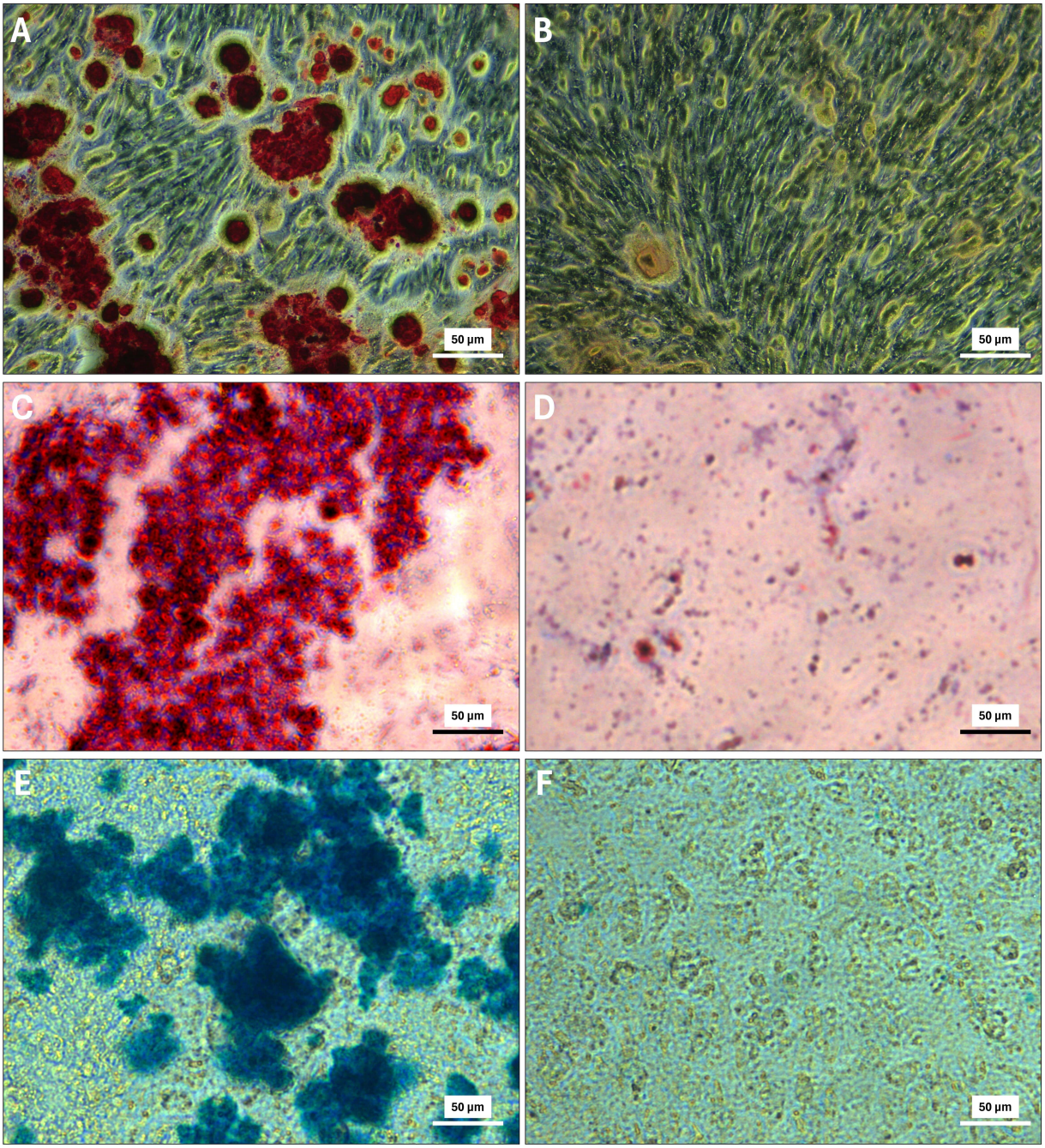

Fig. 2. Trilineage differentiation potential of rabbit AdMSCs. (A) Osteogenic differentiation of AdMSC, forming mineralized matrix nodules (depicted in Alizarin Red staining) indicative of osteoblast activity. (C) Adipogenic differentiation of AdMSCs leads to the accumulation of lipid droplets within the cells, visualized by Oil Red O staining. (E) Chondrogenic differentiation of AdMSCs results in cartilage-like structures, as evidenced by Alcian Blue staining for proteoglycans. (B,D,F) AdMSCs are depicted in their undifferentiated state (control) for osteogenic, adipogenic, and chondrogenic differentiation, respectively.

ability of PF127 hydrogel as a vehicle for delivering cellular therapeutics. However, it's noteworthy that the metabolic activity exhibited a slight decrease compared to AdMSCs cultured in regular media (Fig. 5). These findings indicate the necessity for a topical hydrogel application once every week to maintain optimal metabolic activity and efficacy of the loaded AdMSCs.

Cell Apoptosis Assay

The cell apoptosis assay utilizing DAPI stain is widely employed for the detection and quantification of apoptosis—a programmed cell death process characterized by distinct morphological alterations in the nucleus. DAPI, a DNA-specific fluorescent dye, specifically binds to DNA within the cell nucleus, emitting blue fluorescence upon

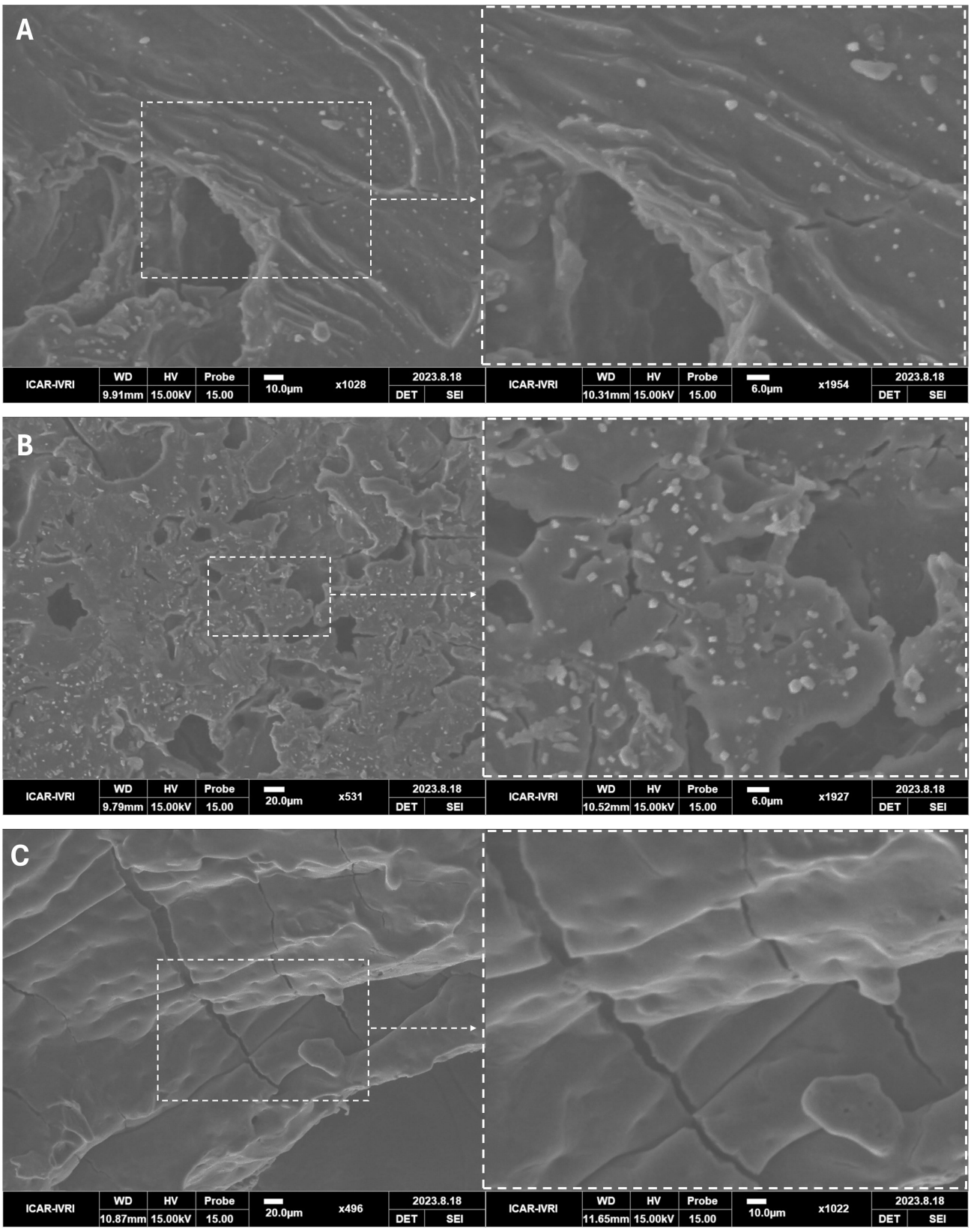

Fig. 3. Scanning electron microscopy (SEM) images of freeze-dried 25% Pluronic F127 hydrogel. (A,B) Channels were observed on the surface indicative of network morphology. (C) Pores were also observed on the surface. The surface of the hydrogels showed interconnected channels with pores, giving an irregular texture.

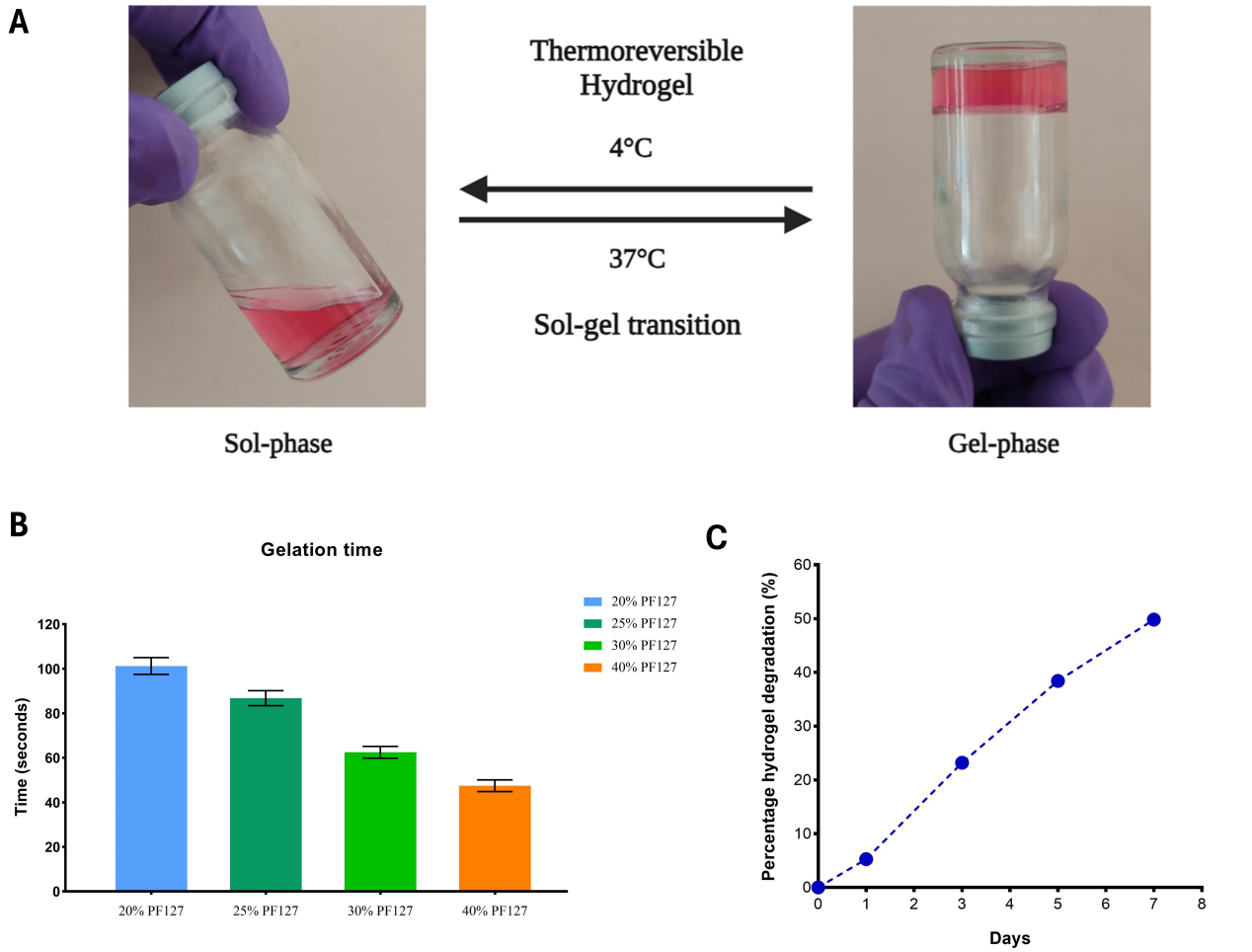

Fig. 4. Physical characteristics of Pluronic F127 (PF127) hydrogel. (A) Reversible thermoresponsive characteristics exhibited by PF127 hydrogel (Sol-Gel transition). (B) Histogram showed the gelation time (mean ± standard deviation (SD) in seconds) of 20% (n = 12), 25% (n = 11), 30% (n = 10), and 40% (n = 10) PF127 hydrogel at physiological temperature (37 °C). (C) The line graph showed a percentage hydrogel degradation (%) of 25% PF127 hydrogel at different time intervals (days 1, 3, 5, and 7) (n = 6).

binding [24,25]. Apoptotic cells typically undergo chromatin condensation and fragmentation, resulting in discernible changes in nuclear morphology. These alterations, such as nuclear condensation and fragmentation, are visualized and quantified through fluorescence microscopy. This assay provides valuable insights into the extent of apoptosis within cell populations subjected to different experimental conditions (Fig. 6).

The outcomes of the apoptosis assay yielded valuable insights into the impact of the experimental treatment (25% PF127) on AdMSCs viability and apoptosis in comparison to the control group (standard culture medium). Apoptosis, being a dynamic process, showcased significant variations in its levels over time. While the control group depicted a modest increase in apoptosis from day 3 to day 7, the experimental group displayed a substantial rise during the same duration. However, despite the higher apoptosis rate observed in the hydrogel group compared to the control after seven days, the crucial point is that the rate remained relatively low. This suggested that a majority of cells within the experimental group (25% PF127) remained viable on day 7. Recognizing the paramount importance of cell viability in tissue regeneration and wound healing, these findings underscored the significance of maintaining viable cells for the repair of damaged tissues.

Discussion

Injectable hydrogels offer several advantages over pre-formed scaffold-based approaches, including high contourability, minimally invasive delivery, and convenient incorporation of therapeutic agents such as drugs and therapeutic cells [26]. Hydrogel-based delivery systems are widely utilized for stem cell delivery, addressing certain limitations inherent in cell-based therapy [27]. Stem cell viability following injection into wounds is often compromised due to factors such as mechanical washout, inadequate extracellular matrix support for binding, leakage, exposure to high shear stresses during injection, and contact with inflammatory mediators and reactive oxygen species

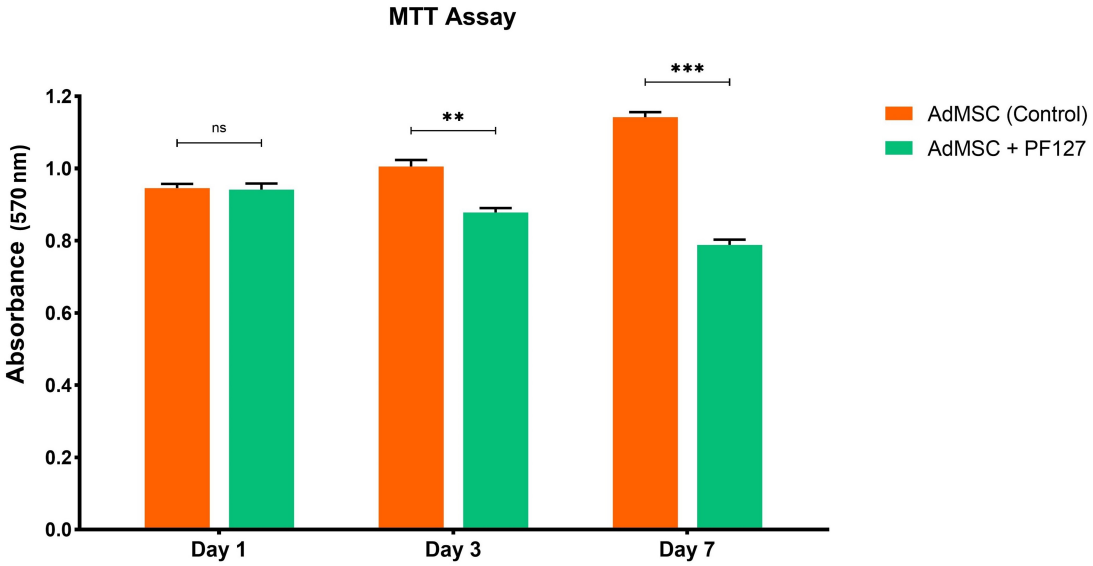

Fig. 5. Evaluation of cytotoxicity using MTT assay. The histogram showed mean ± SD values of MTT assay absorbance in control and Pluronic F127 hydrogel groups on days 1, 3, and 7 (n = 3). Note: ns - non-significant, ** - $p < 0.01$, and *** - $p < 0.001$; MTT, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide.

in the wound environment [28]. Moreover, the absence of adequate cell-supporting signals surrounding the transplanted cells can lead to the eventual demise of stem cells [29]. Notably, the mechanical properties of hydrogels closely resemble those of soft tissue, thus providing substantial support for the healing process [1].

Typically, cells seeded within bioactive scaffolds tend to remain within the scaffold structure for extended periods due to excessive adhesion. Conversely, cells seeded in hydrogels exhibit minimal adhesion to the extracellular scaffold, leading to detachment and migration from the hydrogel matrix towards the wound surface [28]. Consequently, hydrogels serve as a supportive scaffold for delivering therapeutic cells to the wound site without significantly compromising their viability [28,30]. Moreover, they possess adequate permeability to transport therapeutic and signaling factors to the wound area while safeguarding the delivered cells from the host immune system [28]. However, maintaining long-term viability remains a challenge in hydrogel-based cellular therapy due to nutrient and gas deficiencies [31]. Addressing this challenge involves creating hydrogel networks with specific porosity that can facilitate the diffusion of oxygen and other essential macromolecules [31].

The gelation time under physiological conditions signifies the moment when the liquid PF127 solution transitions into a gel state at 37 °C [32]. It represents the duration needed for the sol phase of the hydrogel to convert into its gel phase. This timeframe holds significance in thermoresponsive injectable hydrogel formulations, providing insights into the transformation from a liquid to a gel state, allowing appropriate measures to retain the material at the application site. Gelation time is a pivotal factor influencing the suitability of a hydrogel for various applications. It can impact the ease of handling during preparation, the hydrogel's capacity to encapsulate therapeutic agents, and its potential for sustained drug release.

The selection of PF127 concentration for a specific application should consider the desired gelation time. Higher concentrations might be preferable for applications necessitating rapid gelation, such as wound dressings or minimally invasive drug delivery systems. Conversely, lower concentrations might be more suitable for applications requiring longer working time. Researchers and clinicians can use this information to optimize the formulation of PF127 hydrogels tailored to specific purposes. By adjusting the PF127 concentration, they can customize the gelation time to meet the precise requirements of the intended application.

Stem cell therapy represents a pivotal component of regenerative medicine [33–35]. Yet, beyond just delivering stem cells to the site of injury, an optimal supportive matrix is crucial to nurture the growth and differentiation of transplanted stem cells [36]. Hydrogels have garnered significant attention as scaffolds for delivering stem cells, largely due to their capacity to mimic the native extracellular matrix [36,37].

PF127 stands out as a synthetic hydrogel renowned for its non-toxic, biocompatible, and bioabsorbable properties [11,38]. Moreover, it has garnered approval from the United States Food and Drug Administration (US-FDA) for human use [10,11]. Previous studies have extensively verified PF127's non-cytotoxic nature and its capability to host and support stem cells [10,11,39,40]. Notably, PF127 has served as a scaffold in engineering adipose tissue constructs [11,39].

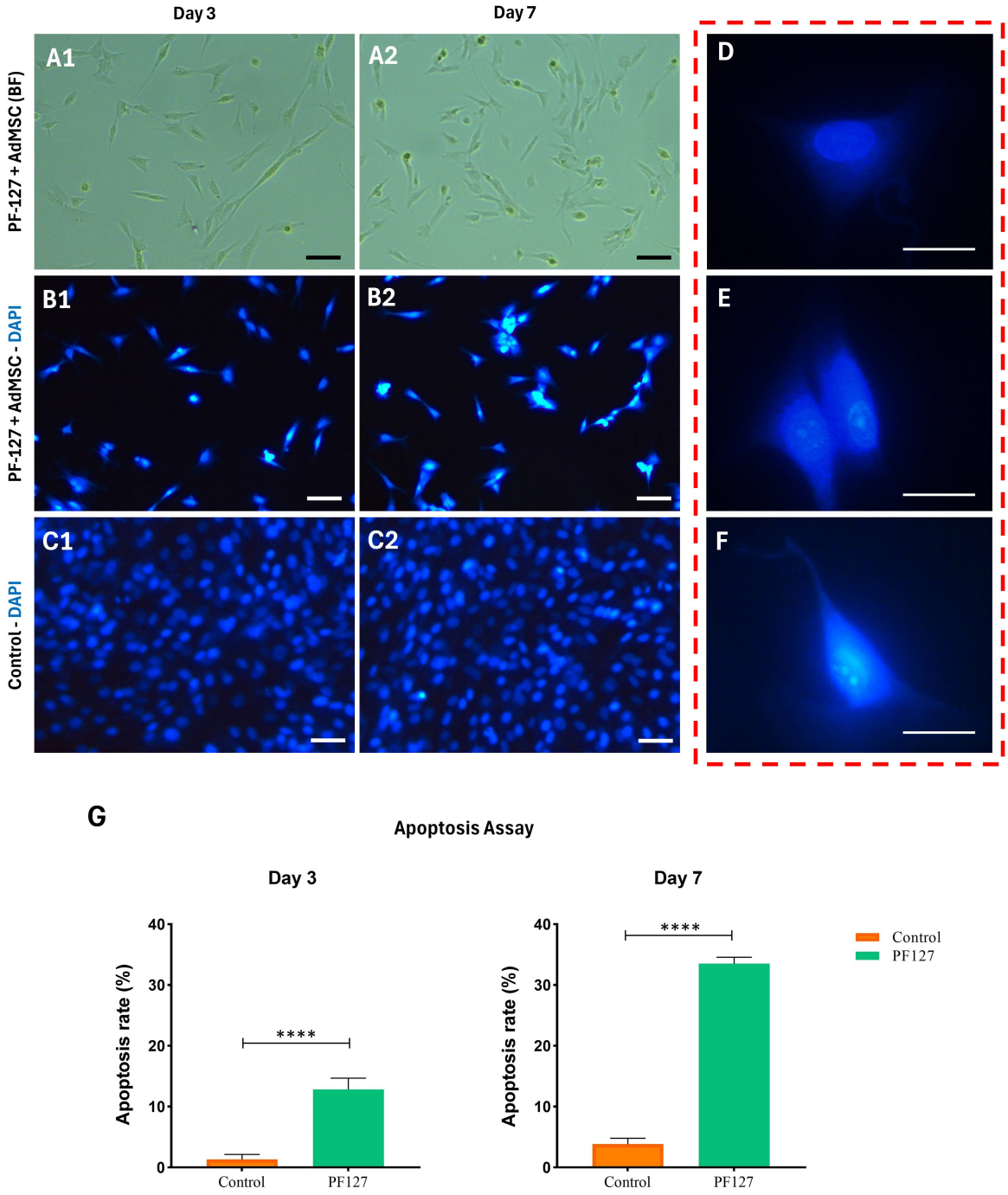

Fig. 6. Cell apoptosis assay using 4′,6-diamidino-2-phenylindole (DAPI) stain comparing AdMSCs loaded in Pluronic F127 hydrogel and normal AdMSCs cultured in culture medium on days 3 and 7 (Scale bar: 20 µm). (A1,A2) AdMSCs loaded PF127 under a bright-field microscope. (B1,B2) DAPI-stained AdMSCs loaded PF127 under a fluorescent microscope. (C1,C2) DAPI-stained AdMSCs in control wells under a fluorescent microscope. Apoptotic cells showed characteristic changes in nuclear morphology, such as nuclear condensation and fragmentation, that could be visualized using fluorescence microscopy (Scale bar: 20 µm). (D) Normal AdMSCs nuclear staining. (E,F) Apoptotic AdMSCs with condensed, bright, and punctate nuclei. (G) Histogram showing mean ± SD values of percentage apoptotic cells (%) in control and PF127 hydrogel groups on days 3 and 7 (n = 6). Note: **** - $p < 0.0001$.

A notable study involved creating a three-dimensional (3D) system using PF127 hydrogel to culture rat bone marrow-derived MSCs [11]. When rat BM-MSCs were loaded into the hydrogel, they displayed adipogenic differentiation when subjected to adipogenic stimuli during a 2-week incubation. Intriguingly, adipogenic differentiation was also observed in the absence of specific stimuli, showcasing the hydrogel's influence on these cells' behav-

ior [11]. Similarly, another study assessed the growth and proliferation of rat bone marrow MSCs cultured in three-dimensional Pluronic F127 gel [39]. When subjected to adipogenic stimuli, the loaded rat BM-MSCs exhibited adipogenic differentiation within the gel. These findings highlighted the potential of 3D Pluronic F127 gel scaffolds to support MSC differentiation into adipocytes, suggesting applications in tissue engineering and repairing fat injuries [39].

Indeed, PF127's non-toxic nature has been observed across various stem cell types, including tooth germ stem cells [41], bone marrow MSCs [10,42], dental pulp stem cells [10], and umbilical cord MSCs [43].

Specifically, when umbilical cord MSCs were encapsulated within PF127 hydrogel, they showcased the potential to restore the morphology of a simulated thin endometrium, suggesting a role in endometrium regeneration [43]. This restoration was characterized by increased endometrial thickness, a higher count of glands, and enhanced neovascularization capacity, underscoring the significant potential of PF127-based hydrogel systems in fostering tissue regeneration within the endometrial environment [43].

Certainly, PF127's versatility extends to various therapeutic strategies involving cartilage regeneration and wound healing. It has been utilized as a delivery vehicle for BM-MSCs in articular joint therapy, aiming to promote cartilage regeneration in osteoarthritis models [44]. This approach involves inducing the chondrogenic differentiation of the transplanted BM-MSCs, potentially aiding in cartilage repair within osteoarthritic joints. Moreover, beyond direct loading of AdMSCs into PF127 hydrogel, 3D bioprinting techniques have been explored to generate cell-laden scaffolds using PF127. These scaffolds are designed to promote wound healing by fostering the osteogenic differentiation of apical papilla stem cells under low voltage–frequency electromagnetic fields [45]. The 3D bioprinted PF127 hydrogels serve as excellent matrices for encapsulating stem cells, facilitating controlled cell delivery and providing an environment conducive to tissue regeneration [45].

PF127's non-toxic nature makes it an ideal candidate for various sustained-release applications, such as delivering drugs, growth factors, or cells into the body without causing adverse effects on physiological functions [46]. Studies have shown that PF127 hydrogel can serve as a carrier for MSC-derived paracrine factors, contributing to synergistic biological effects [47,48].

Moreover, the combination of PF127 hydrogel with other materials such as folic acid/MgO:ZnO/chitosan hybrid particles has been explored to create a 3D delivery system for loading AdMSCs [48]. This composite scaffold exhibits promise for wound regeneration by providing structural support and enhancing the viability of encapsulated AdMSCs at the injury site [48]. The composite scaffold's ability to support cell viability and provide a conducive environment for wound healing indicates its potential as a therapeutic tool in tissue regeneration applications.

Indeed, research involving rabbit BM-MSCs loaded into PF127 composite hydrogel containing TGF-β3 has demonstrated the ability to induce osteoblastic differentiation [40]. Moreover, the scaffold's potential to trigger osteogenesis and angiogenesis has been corroborated in the rabbit maxillary sinus lifting model [40]. Similarly, investigations into human dental pulp stem cells (DPSCs) and human bone marrow-derived mesenchymal stem cells (hBM-MSCs) within the PF127 hydrogel scaffold have revealed substantial osteogenic and adipogenic gene marker expression after two weeks of differentiation [10]. These findings support PF127's potential in directing stem cell differentiation and indicate its utility in tissue engineering for cell delivery purposes due to the high viability and proliferation observed with encapsulated DPSCs and hBM-MSCs [10].

Overall, the versatility of Pluronic F127 hydrogel as a scaffold for delivering cellular therapeutics, particularly mesenchymal stem cells, has been highlighted in multiple studies [10,11,39,40]. Its capacity to induce differentiation and maintain cell viability underscores its significance in tissue engineering and regenerative medicine [40].

The DAPI stain is commonly utilized to assess apoptosis due to its ability to bind to DNA and exhibit distinctive fluorescence when observed under a fluorescent microscope. When cells undergo apoptosis, there are notable changes in the nuclear morphology that can be visualized with DAPI staining [23,24]. The normal nucleus typically appears uniformly stained with DAPI, emitting a diffuse blue fluorescence [25]. However, during apoptosis, the nucleus undergoes changes such as condensation, fragmentation, and blebbing, resulting in altered patterns of DAPI staining.

Under fluorescence microscopy with appropriate DAPI filters (excitation around 358 nm and emission approximately 461 nm), apoptotic cells show distinct characteristics. These include intensified blue fluorescence due to increased DNA condensation and fragmentation within the nucleus. Additionally, nuclear blebbing, a characteristic feature of apoptosis, can be visualized as distinct protrusions or irregularities on the nuclear membrane. These alterations in nuclear structure and intensity of DAPI staining serve as indicators for identifying and quantifying apoptotic cells in a given cell population.

Conclusions

The investigation into PF127 hydrogel highlights its reversible thermoreversible behavior, ideal network morphology, breathability, and rapid degradation profile. These properties make PF127 a promising candidate for various applications, including wound healing, drug delivery, and tissue engineering. Furthermore, the cytotoxicity analysis and cell apoptosis assay emphasize the bio-

compatibility and viability of AdMSCs when loaded into PF127 hydrogel, even though there is a slight decrease in metabolic activity compared to standard culture media. The ability to control gelation time by adjusting PF127 concentration is a significant advantage, as it allows for tailored use in different applications. Overall, this research underscores the potential of PF127 hydrogel as a supportive matrix for AdMSCs, highlighting its role in regenerative medicine. The combination of AdMSCs and PF127 may pave the way for innovative therapeutic strategies and applications in tissue repair and regeneration. The study's findings contribute to the ongoing exploration of stem cell-based therapies and biomaterials, offering new possibilities for improving patient outcomes and advancing the field of regenerative medicine.

Availability of Data and Materials

Datasets supporting the conclusions of this article are included within the manuscript.

Author Contributions

KS: conception and design, data collection, analysis and interpretation of data, and writing the initial draft and critical revision. SAB, MM, RSE, RK, ORV, AMP, KD, SKM and AP: study design, analysis and interpretation of data and critical revision. All authors approved the submitted version and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All experimental protocols used in the study were approved by the Institutional Animal Ethics Committee (IAEC), ICAR-Indian Veterinary Research Institute, vide order No. 26-1/2022-23/JD(R) under protocol no. IAEC/07.07.2022/S31.

Acknowledgment

The authors thank the Director, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly, India, and the All-India Network Program on Diagnostic Imaging and Management of Surgical Conditions in Animals (AINP-DIMSCA) for providing the necessary research facilities to carry out this work. The authors also acknowledge the help provided by Dr. Yasotha Thirupathi, Dr. Sushmita Nautiyal, and Dr. Gaurav Sharma.

Funding

This research received no external funding.

Conflict of Interest

The authors declare no conflict of interest.

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