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Exosome-loaded alginate hydrogels as modulators of B16-F10 melanoma cell migration

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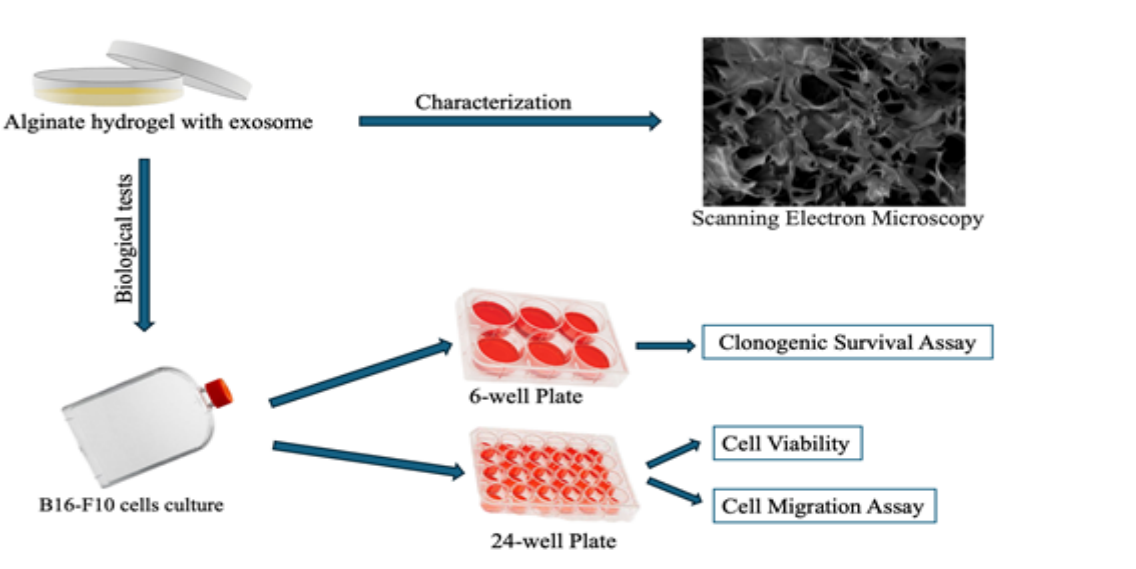

Abstract: Exosomes have gained attention as promising therapeutic agents in cancer treatment due to their ability to influence target cell phenotypes and modulate immune responses. Their role in tumor biology, however, is influenced by several factors, including the source of mesenchymal stem cells (MSCs), culture conditions, and the tumor microenvironment. This study aimed to evaluate the effects of exosomes derived from bone marrow MSCs of Sprague-Dawley rats, incorporated into alginate hydrogels (AH), on the migration and viability of murine melanoma (B16-F10) cells. Scanning electron microscopy revealed that the hydrogels preserved their structural integrity after exosome incorporation. Both AH and exosome-loaded AH (AHE) exhibited no cytotoxic effects, as the viability and colony-forming capacity of B16-F10 cells remained comparable to untreated controls. Notably, AHE significantly suppressed tumor cell migration, a critical step in cancer metastasis, whereas AH alone had no effect. These findings indicate that exosomes retained their functionality within the hydrogel matrix, effectively modulating cell migration. This study underscores the therapeutic potential of exosome-loaded hydrogels in regulating cancer cell behavior. Nonetheless, further research is needed to elucidate the molecular mechanisms involved and optimize the clinical application of exosome-integrated hydrogels.

Keywords: Exosomes. Alginate hydrogels. Cancer therapy. Cell migration. Cell viability.

Introduction

The term "cancer" encompasses a group of over 100 diseases characterized by complex biological processes, including disruptions in cell cycle regulation, loss of cellular function, and the migration and invasion of mutated cells into other tissues[1,2]. The primary mechanisms underlying carcinogenesis involve mutations that activate oncogenes and deactivate tumor suppressor genes. These mutations may arise from endogenous processes, such as errors during cell replication, genetic inheritance, chronic inflammation, and oxidative stress, or from exogenous factors, such as exposure to mutagenic or genotoxic agents[3, 4].

In Brazil, an estimated 704,000 new cancer cases are expected to occur between 2023 and 2025, with 483,000 cases excluding non-melanoma skin cancer, the most prevalent type in the country, representing approximately 30% of all registered malignant tumors[5].

Focusing on skin cancer, melanoma accounts for approximately 3% of malignant neoplasms of the skin and poses the highest risk for metastasis, making it the most severe form of the disease[1,2]. Originating from melanocytic lineage cells, melanoma is characterized by an invasive growth pattern and early dissemination. While endogenous risk factors, such as fair skin and a genetic predisposition to multiple nevi, contribute to its development, ultraviolet light exposure remains the most significant exogenous risk factor[6].

Melanoma treatments include surgical excision of the affected area, with radiotherapy and chemotherapy being standard approaches for advanced stages[5]. However, these treatments are associated with significant toxicity and adverse effects, driving the need for novel therapeutic strategies.

Exosomes, a class of extracellular vesicles (EVs) of endosomal origin and nanometric dimensions, are mediators of intercellular communication under both physiological and pathological conditions. These vesicles can contain DNA, RNA, lipids, metabolites, and cytosolic and cell surface proteins[7]. Studies have revealed that molecular content of exosomes derived from healthy cells differs significantly from those of cancer cells[8].

In the context of cancer, exosomes can modulate the tumor microenvironment, influencing processes such as immunosuppression, tumor progression, and responses to existing therapies[7,9]. Exosomes can express molecules involved in immunosuppression, such as PD-L1 and transforming growth factor-β (TGF-β)[7,10]. Furthermore, exosomes from dendritic and tumor cells have been found to express class I major histocompatibility complex (MHC I) molecules and tumor markers, such as heat shock proteins (HSPs). These molecules are implicated in antigen presentation and the activation of T cells, enabling CD8+ T cell-dependent antitumor responses in both in vitro and in vivo studies[11, 12].

Given these findings, this study aims to investigate the effects of hydrogel therapy containing mesenchymal stem cells (MSCs)-derived exosomes on the in vitro proliferation and migration of murine melanoma (B16-F10) cells. This research seeks to contribute to the understanding of biological responses associated with exosome-based therapies, providing a scientific foundation for future studies exploring innovative approaches to cancer treatment.

Material and Methods

Alginate hydrogels with exosomes

Exosomes were isolated from the conditioned medium of bone marrow-derived mesenchymal stem cells (MSCs) obtained from Sprague-Dawley rats using size-exclusion chromatography (Izon qEVsingle SEC columns, Boston, USA), following the globally recognized methodology detailed in previous studies[13,14]. The resulting exosome samples were subsequently stored at -80 °C to preserve their integrity. These exosomes were incorporated into alginate hydrogels prepared using a 1.5% (w/v) sodium alginate solution dissolved in water. The exosome suspension (25 µL) was added to the alginate solution (200 µL) at room temperature, followed by crosslinking with a 0.1% calcium chloride ($CaCl_2$) solution to form the hydrogel[15]. The hydrogels were designated as AHE (hydrogels containing exosomes) and AH (alginate hydrogels without exosomes), with the latter serving as a control in the biological assays. The surface morphology of the hydrogels was analyzed with a scanning electron microscope (FEI Inspect S 50, at the Laboratory of Structural Characterization, UFSCAR, Brazil). The hydrogels were prepared and freeze-dried at -50°C to remove any remaining moisture. Afterward, they were sectioned and coated with a thin layer of gold to enhance conductivity. The microstructural evaluation was performed at an acceleration voltage of 2.0 kV. Rheological tests were conducted using an Anton Paar RheoCompass MCR-92 rheometer to evaluate the influence of the exosomes incorporation in the viscosity of AH. The tests were performed at 25°C with a plate geometry of 50 mm in diameter and a gap of 0.2 mm. Viscosity measurements were carried out over a shear rate range of 1 to 100 s^{-1}.

Cell Line and Culture Conditions

The murine melanoma cell line B16-F10 (ATCC®

CCL-6475™) (Figure 1) was obtained from the Rio de Janeiro Cell Bank (BCRJ) and cultured in Dulbecco's Modified Eagle Medium (DMEM, Sigma Aldrich), supplemented with 10% fetal bovine serum (FBS - Gibco). Incubation conditions were maintained at 37°C with 5% CO_2 and 95% humidity. During the experimental period, the cell line was subcultured by trypsinization upon reaching confluence.

Cell Viability - Resazurin Reduction Assay

The viability of the B16-F10 cells was evaluated using the metabolic indicator resazurin (7-hydroxy-3H-phenoxazin-3-one 10-oxide, Sigma Aldrich Corp., St. Louis, MO, USA). The assessment was conducted following 48-hour treatments with AH and AHE (200 µL). The cells were seeded in 24-well plates at an initial density of 1 × 10^5 cells/well. The cells were incubated under standard culture conditions for 24 hours to allow cell adhesion, the formation of a semi-confluent monolayer, and progression into the exponential growth phase. Untreated cells cultured in complete medium served as the negative control, while cells exposed to 50% dimethyl sulfoxide (DMSO) were used as the positive control for cytotoxicity. After the treatment period, 0.01% (w/v) resazurin was added to each well for analysis[16]. Fluorescence measurements were obtained using a Cytation (Biotek®) microplate reader with excitation and emission filters of 530 nm and 590 nm, respectively. The fluorescence of untreated cells, serving as the negative control, was considered as 100%.

Clonogenic Survival Assay

B16-F10 cells were seeded in 6-well plates at an initial concentration of 1 × 10^5 cells/well in 2 mL of complete culture medium and incubated under standard culture conditions for 24 hours. Following incubation, the cells were treated with AH and AHE (400 µL) for 24 and 48 hours. Untreated cells cultured in complete DMEM medium served as the negative control. At the end of the treatment period, the cells were washed, trypsinized, and counted using a Neubauer chamber to obtain a suspension containing 200 cells/well. These cells were reseeded in 6-well plates and incubated under standard conditions to allow colony formation from individual cells. After seven days of culture, the colonies were washed, fixed with a methanol:acetic acid:distilled water solution (1:1:8) for 30 minutes, and stained with 2 mL of Giemsa stain (Sigma-Aldrich) diluted in phosphate buffer (1:20) for 20 minutes. After staining, the colonies were washed with distilled water and counted[17]. The number of colonies in the negative control was considered 100%, and survival fractions (SF) for each treatment were calculated as equation (1):

$$SF\ (\%) = \frac{\text{Number of colonies counted in each treatment}}{\text{Number of colonies counted in the negative control}} \times 100 \quad (1)$$

Cell Migration Assay

For the cell migration assay (scratch assay), B16-F10 cells were seeded at a density of 1 × 10^6 cells/well in 24-well culture plates. A cell-free area was created by scratching the monolayer with a sterile 200 µL pipette tip. The culture medium was then replaced to remove cellular debris, and the cells were treated with AH and AHE (200 µL). Cell migration into the cell-free area was monitored at 24 and 48 hours. Microphotographs were captured using a digital camera (DFC7000T) mounted on an optical microscope (Leica DMi8, Frankfurt, Germany). The migration was quantified using ImageJ analysis software (NIH, Bethesda, MD, USA) and normalized to the initial cell-free area at time 0[18].

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 7.0 software (GraphPad Software Inc., San Diego, CA, USA). A one-way analysis of variance (ANOVA) was used to evaluate statistical significance, followed by Tukey's post hoc test, with a significance threshold set at $p < 0.05$. All experiments were conducted in triplicate, and statistical significance was assessed by comparing treatment responses to the negative control.

Results

The microstructures of AH and AHE, as well as the results of the viscosity analysis by rheology, are presented in Figure 1. The SEM images reveal that the hydrogel (AH) exhibits a uniform and highly porous inner structure (Fig. 1A). Incorporation of the conditioned medium containing exosomes led to morphological changes in the hydrogel, resulting in a reduction in pore size (Fig. 1B). Exosomes are visibly embedded within the hydrogel's porous network (arrow, Fig. 1B), confirming their successful incorporation while preserving the overall architecture of the hydrogel. A magnified view of the region indicated by the arrows in Fig. 1B highlights the presence of exosomes integrated into the AH structure (Fig. 1C). Although the spherical shape of the exosomes is not preserved due to the freeze-drying process, the images demonstrate the integrity of the exosomes, with no damage to their

external membrane. Furthermore, the incorporation of exosomes into the AH matrix significantly increased its viscosity, as demonstrated in Fig. 1D. The higher viscosity of AHE compared to AH corroborates the denser structure of the exosome-enriched hydrogel.

The results of the cell viability, assessed by the resazurin assay, and the survival fractions obtained from the long-term cytotoxicity evaluation using the clonogenic survival assay, showed no statistically significant differences when compared to the negative control. Neither AH nor AHE induced a reduction in the viability of B16-F10 cells (Fig. 2), nor did they inhibit colony formation (Fig. 3), regardless of the treatment duration (24 or 48 hours). These findings indicate that neither AH nor AHE exhibited cytotoxic effects on the B16-F10 cell line under the tested conditions. However, the scratch assay revealed that AH did not affect the migration of B16-F10 cells. In contrast, treatment with AHE significantly inhibited the migration of B16-F10 tumor cells compared to the negative control (culture medium supplemented with 10% FBS), with this effect being evident at the 48-hour time point (Fig. 4).

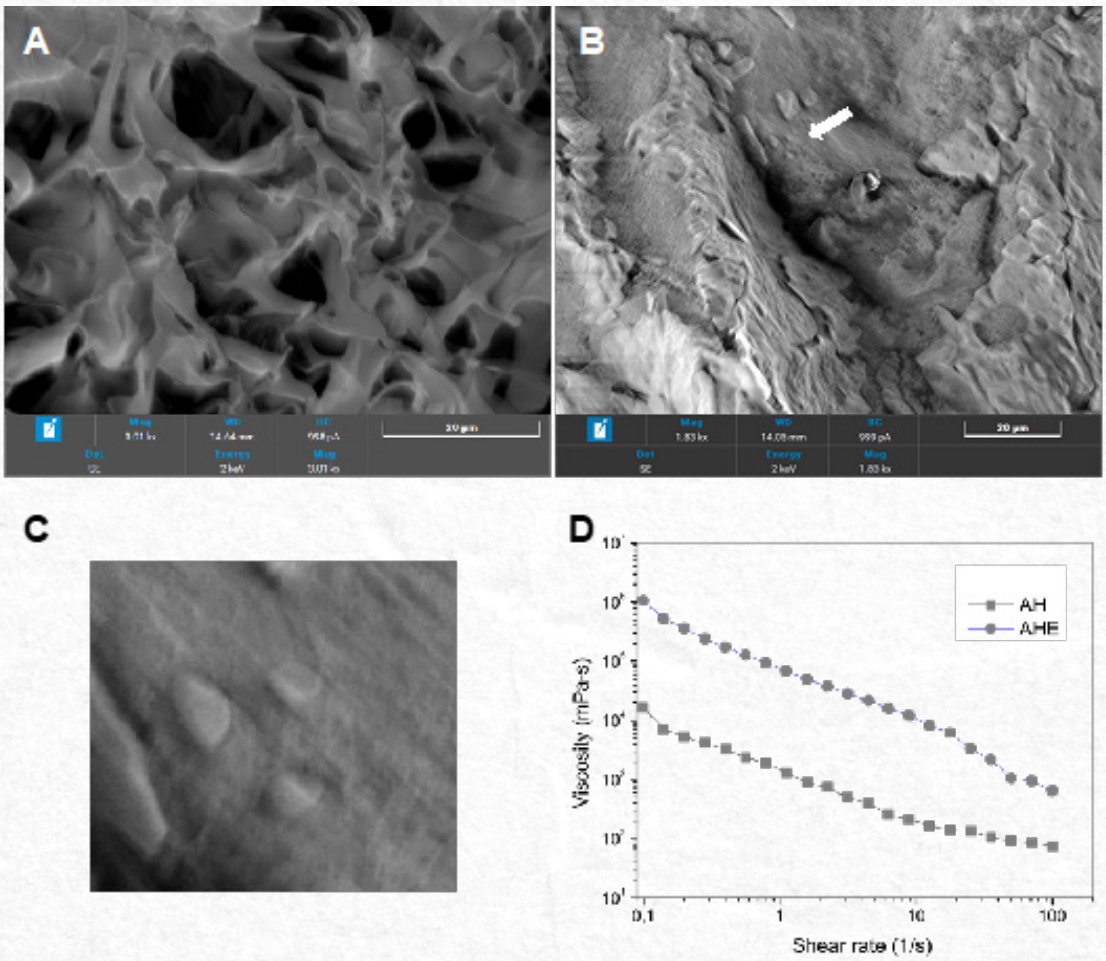

Figure 1 - Scanning Electron Microscopy (SEM) images of AH (A), AHE (B), a magnified view of the region in (B) indicated by arrows, highlighting the exosomes within the hydrogel structure (C), and the results of the viscosity analysis (D).

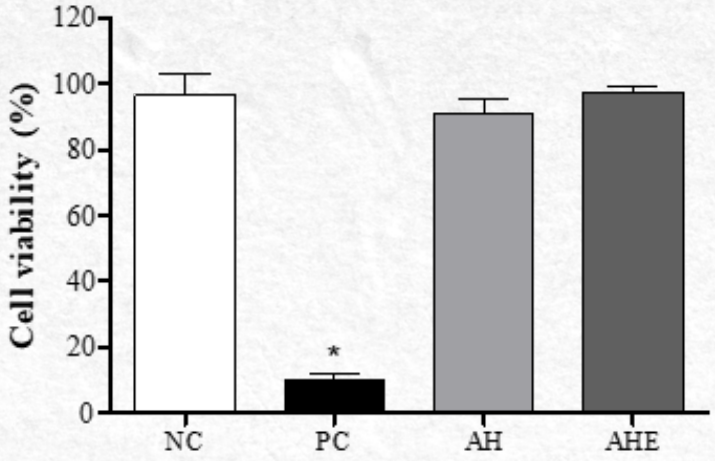

Figure 2 - Cell viability (%) of B16-F10 cells after 48-hour treatment with sodium alginate hydrogel incorporated (AHE) or not (AH) with exosomes.
NC: negative control (DMEM with 10% fetal bovine serum, 100% cell viability); CP: positive control (dimethyl sulfoxide 50%); Results were expressed as mean ± standard deviation of three independent assays. *Statistically different from NC ($p<0.05$, ANOVA, followed by Tukey's test).

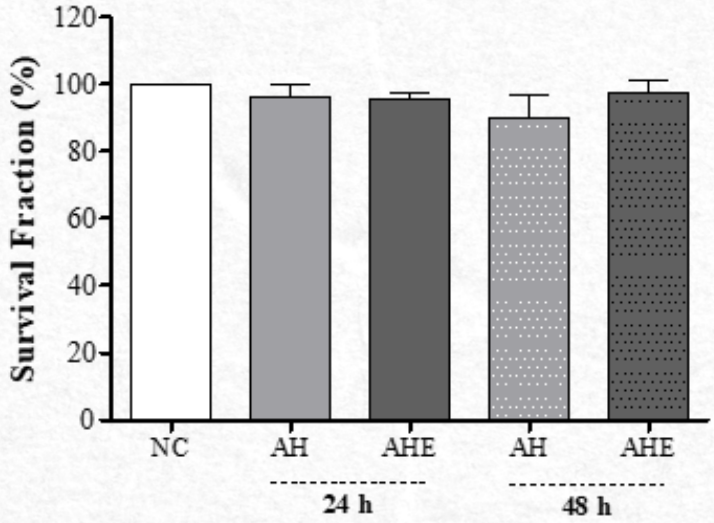

Figure 3 - Survival fraction (%) of B16-F10 cells after 24 and 48-hour treatment with sodium alginate hydrogel incorporated (AHE) or not (AH) with exosomes.
NC: negative control (DMEM with 10% fetal bovine serum, 100% cell viability). Results were expressed as mean ± standard deviation of three independent assays. *Statistically different from NC ($p<0.05$, ANOVA, followed by Tukey's test).

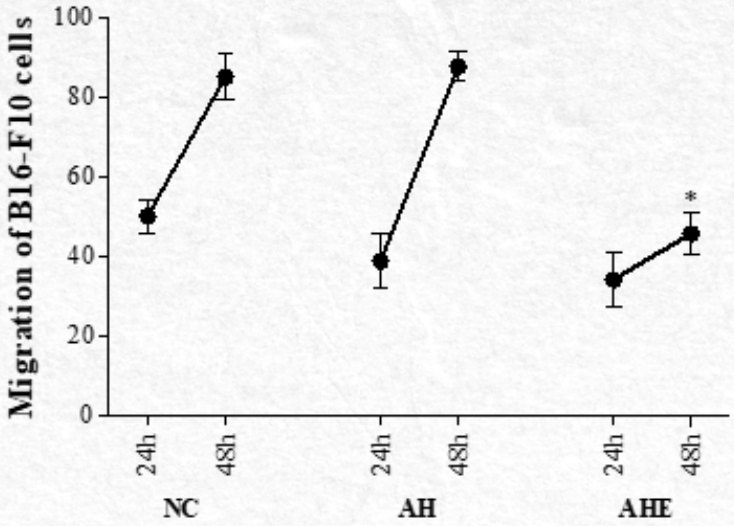

Figure 4 – Percentage of migration of B16-F10 cells treated with sodium alginate hydrogel incorporated (AHE) or not (AH) with exosomes.
The cell-free zone at the designated study points was normalized in relation to that obtained at 0 h. NC, negative control (culture medium with 10% fetal bovine serum). *Statistically different from NC ($p<0.05$, ANOVA, followed by Tukey's test).

Discussion

Exosomes have emerged as promising candidates in cancer therapy due to their ability to modulate immune responses and transfer molecular cargos that can influence the phenotype of target cells. However, as these vesicles participate in various cellular processes, they possess a dual nature in cancer biology as paracrine mediators, capable of either promoting or suppressing tumor progression[12,19].

Studies show that while exosomes derived from MSCs have been shown to facilitate tumor progression by transferring signaling molecules such as miRNAs, which can activate pathways like ERK1/2 in solid tumors[20-23], they can also exert anti-tumor effects by delivering miRNAs, proteins, and long noncoding RNAs (lncRNAs) that suppress cancer cell proliferation and induce apoptosis[24-30]. These contrasting effects underscore the complexity of MSC-derived exosomes in cancer therapy, which are influenced by factors such as the source of MSCs, the culture conditions, exosome extraction methods, and the tumor microenvironment[19].

Given this complexity, it is crucial to standardize protocols for MSC culture and exosome isolation to fully elucidate their effects on specific cancer types[19]. In this context, our study investigated the effects of exosomes from bone marrow-derived MSCs embedded in alginate hydrogels (AH), particularly in the modulation of cell migration and viability, on murine melanoma (B16-F10) cells.

Our findings demonstrate that the alginate hydrogels (1.5% w/v) used in this study acted as a platform for the incorporation of exosomes, offering a protective environment that may contribute to their stability. Rheological analysis revealed that the incorporation of exosomes increased the viscosity of the hydrogel, indicating a denser and more cohesive structure. This suggests that the hydrogel matrix was capable of integrating exosomes, preserving its ability to act as a delivery system. The rapid degradation of the alginate hydrogels makes them particularly suitable for short-term studies, facilitating the release of exosomes while preserving their biological activity. This indirect evidence, supported by both rheological and structural findings, suggests that the functional integrity of the exosomes was maintained during incorporation and release. However, further molecular or functional assays are needed to comprehensively confirm the stability and integrity of exosomes within this system.

In our experiments, we observed that both AH (alginate hydrogel) and AHE (hydrogel with exosomes) did not induce cytotoxicity in B16-F10 cells. The viability and colony-forming ability of the cells treated with AH and AHE remained comparable to the untreated control group, indicating that the

exosomes did not adversely affect the proliferative capacity of these cells.

However, a key finding in our study was the significant inhibitory effect of AHE on the migration of B16-F10 tumor cells. Cell migration is a critical process in physiological functions like tissue development, immune response, wound healing, and cancer metastasis, the latter being a hallmark of malignant tumors[31]. While AH did not influence the migration of melanoma cells, the inclusion of exosomes in the hydrogel (AHE) effectively suppressed cell movement, highlighting the potential of MSC-derived exosomes in regulating tumor cell migration.

Studies have demonstrated that exosomes derived from MSCs can modulate key factors involved in cancer progression, including angiogenesis. For instance, MSC-derived exosomes have been shown to reduce angiogenesis by downregulating vascular endothelial growth factor (VEGF) and CD31, both critical markers of angiogenesis[32,33].

In a study by Pakravan et al.33, the exosomal transfer of miR-100 from MSCs into breast cancer cells led to a dose-dependent decrease in VEGF expression, effectively suppressing angiogenesis in vitro. This suppression was mediated through modulation of the mTOR/HIF-1α signaling axis in breast cancer cells. Furthermore, conditioned media from breast cancer cells stimulated with MSC exosomes resulted in decreased endothelial cell migration and proliferation, reinforcing the role of MSC exosomes in inhibiting angiogenesis. miR-100, a tumor-suppressive microRNA, is typically downregulated in various breast cancer subtypes. Bone marrow-derived MSC exosomes are particularly enriched with miR-100, which may contribute to their anti-tumorigenic effects.

Other studies have shown that MSCs overexpressing miR-146b secrete exosomes loaded with miR-146b. miR-146b targets EGFR (epidermal growth factor receptor) mRNA and downregulates EGFR and NF-κB in glioma cells, which leads to inhibition of cell migration and invasion. Moreover, this exosomal delivery of miR-146b resulted in reduced tumor volume in vivo[34]. Similarly, exosomal delivery of miR-143 from human bone marrow-derived MSCs inhibited the migration of osteosarcoma cells, specifically the 143B cell line[35]. In most cases, exosomes induced cell cycle arrest in the G0/G1 or G2/M phases, limiting tumor cell proliferation[36].

While the mechanisms through which MSC exosomes inhibit migration of B16-F10 melanoma cells remain unclear, existing studies offer insight into potential pathways. For example, Otsu et al.[37] demonstrated in an in vivo melanoma model that MSC injection resulted in reduced expression of endothelial cell markers and decreased tumor vasculature. Similarly, Maestroni et al.[38] found that co-injection of bone marrow MSCs with tumor cells in C57BL/6 mice inhibited tumor growth and metastasis formation in Lewis lung carcinoma (LLC) and B16 melanoma cell lines. These studies suggest that MSC exosomes may play a pivotal role in modulating tumor progression and metastasis through various cellular and molecular mechanisms.

Conclusion

Our findings demonstrated that exosomes derived from bone marrow MSCs incorporated into alginate hydrogels effectively inhibited the migration of B16-F10 melanoma cells without affecting their viability, suggesting a promising role in controlling tumor metastasis. These results underscore the need for further studies to clarify the molecular pathways involved and to optimize the clinical application of exosome-integrated hydrogels in cancer treatment.

Declarations

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgments

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Author's contributions

All authors discussed the results and contributed to the final manuscript. L.H.D.S. wrote the manuscript with support from F.A.R. L.H.D.S and J.A.P. designed, performed the experiments, analyzed the data. J.F.F isolated and provided the exosomes. E.T. prepared and characterized the hydrogels. F.A.R supervised the project.

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