Harnessing Exosome-Mediated Signaling for Enhanced Bone Regeneration: Novel Insights into Scaffold Design

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INTRODUCTION: There is an increasing need for bone tissue regeneration due to bone injuries like fractures, trauma, infections, osteomas, and revision surgeries. Nevertheless, acquiring conventional bone grafts poses significant difficulties to procure, frequently leading to the recipient's body rejecting the donor. Synthetic grafts are an effective alternative, but their usefulness is limited since they do not accurately imitate the mechanical strength, porosity, bioactivity (especially vascularization), and biocompatibility of actual bone [1]. Considering this, during the last ORS meeting, we presented a novel idea - the utilization of an interpenetrating polymeric network (IPN) scaffold to develop artificial bone [2]. The concept presented integrates natural and synthetic polymers' advantageous characteristics, demonstrating an innovative and promising methodology. The present project explores the potential of tissue rejuvenation and repair through exosome-mediated signaling, with the ultimate goal of facilitating improved bone regeneration [3]. We hypothesized that an IPN scaffold consisting of a novel combination of pectin, chitosan, and exosomes derived from mesenchymal stem cells would be an efficient biomaterial for bone tissue grafting. Therefore, we fabricated a pectin-based IPN scaffold, isolated exosomes from bone marrow-derived human mesenchymal stem cells (hMSCs), and analyzed the physical and chemical properties, porosity, and swelling characteristics. Furthermore, the scaffold's mechanical strength, bioactivity, and *in vitro*, osseointegration properties will also be evaluated *in vivo*.

METHODS: (i) Scaffold fabrication and characterization: The scaffolds were fabricated by a physical crosslinking of 1% w/v polymeric blend of (2:1:1 w/v molecular ratios of Pectin: PVA: Chitosan) by magnetic stirring followed by lyophilization. (ii) Exosomes isolation and characterization: Human mesenchymal stem cells (HMSCs) were cultured until reaching 80% confluency in T75 flasks and subjected to a 2 or 4-week incubation period in either growth or osteogenic differentiation media, as per the specific requirements of the experiment. The exosomes isolated from a 2-week culture of human mesenchymal stem cells (HMSCs) were explicitly used in a 2D culture experiment. To obtain exosomes, cells were exposed to a serum-free media, either for growth or differentiation purposes, for 48 hours. The isolation of exosomes from the conditioned medium was conducted using ExoQuick-TC, an exosome isolation reagent manufactured by System Biosciences. The isolated exosomes were reconstituted in phosphate-buffered saline (PBS), resulting in a suspension of exosomes in a volume of 10µL of PBS for every 10 mL of medium. The concentrations of exosomes were standardized by normalizing them to the cell count of the tissue culture plate from which they originated**.** (iii) *In vitro* cell culture assays: The isolated exosomes were incorporated into the scaffolds (30 µl of exosomes per 10,000 cells) according to the type of assays and used for the assays. More specifically, the osseointegration and osteoconduction properties were evaluated using human mesenchymal stem cells (hMSCs), which can classify the cells by their viability (alamarBlue assay), the number of live cells to dead cells (live-dead stain). SEM analyses were performed to observe the cell adhesion efficiency of the scaffolds in the presence of exosomes. In addition, the osteoconductive and osteogenic differentiation potential was determined using the alkaline phosphatase activity and RT-PCR analysis for a period of 3,5,7 & 10 days. All the experiments were performed in triplicates. These procedures were adopted to determine the effectiveness of the exosome-mediated IPN scaffold in an actual bone tissue application.

RESULTS: The IPN scaffolds were successfully fabricated using the freeze-drying method. SEM image (Fig 1a) demonstrated the highly interconnected, porous nature of the fabricated IPN scaffold. The pore diameter of the scaffold was calculated using ImageJ analysis and found to be 250 µm, which lies in the range suitable for efficient bone implants. The Exosomes were successfully isolated and characterized using DLS, TEM, and immunoblotting techniques. From the DLS results (Fig 1b -i), the size of exosomes was around 100 nm and was corroborated with TEM micrographs (Fig 1b -ii). *In vitro* cytocompatibility (Fig 1c) shows that the exosomes incorporated IPN scaffold possesses 1-fold % cell viability higher than that of the pectin IPN scaffold, proving the scaffold is non-toxic and the presence of exosomes facilitates cell proliferation effectively. Live/Dead staining (Fig 1d (i &ii)) corroborates the Alamar blue assay and proves the viability of cells is higher with the exosomes incorporated with scaffolds compared to the IPN scaffolds. Fig 1 d (iii & iv) demonstrates the firm attachment of hMSCs onto the scaffolds, confirming the scaffold's osteoinductive nature of recruiting MSCs into osteoblasts that facilitate bone formation. Osteogenic differentiation is analyzed by the ALP activity (Fig 1e), which showed improved ALP activity of around 2.5-fold and a 6-fold increase in the presence of exosomes incorporated scaffold over the period of 7 and 14 days compared to the Pectin scaffold. RT-PCR analysis (Fig 1f) shows the increased gene expression levels in the exosomes, which signifies that osteogenic differentiation is more effective by exosome signaling. Statistical analyses were performed using GraphPad Prism Software, and the results were assessed with ordinary two-way ANOVA followed by a Tukey post hoc test. A value of $p < 0.05$ was considered significant.

DISCUSSION: Exosomes have gained significant interest in the field of regenerative medicine in recent years due to their ability to enhance tissue regeneration, specifically in the field of bone tissue regeneration. Exosomes contain a variety of bioactive chemicals, including growth factors, cytokines, and other genetic elements. These molecules play a crucial function in cellular signaling, consequently having a substantial impact on adjacent cells to enhance the process of osteogenesis. The research findings presented in our study emphasize the effectiveness of exosome-mediated pectin-based IPN scaffolds as a persistent biomaterial with great potential for enhancing the field of bone tissue engineering. The *in vitro* investigations have confirmed that the integration of exosomes into the IPN scaffolds induces a more robust augmentation of bone cell adhesion, mineralization, migration, and proliferation. Hence, exosomes can potentially be utilized as specific carriers for delivering therapeutic medicines for the treatment of bone cancer. However, it is crucial to recognize that the depth of this study is limited due to the lack of *in vivo* animal investigations, which are essential for determining the effectiveness of this strategy in correcting bone abnormalities.

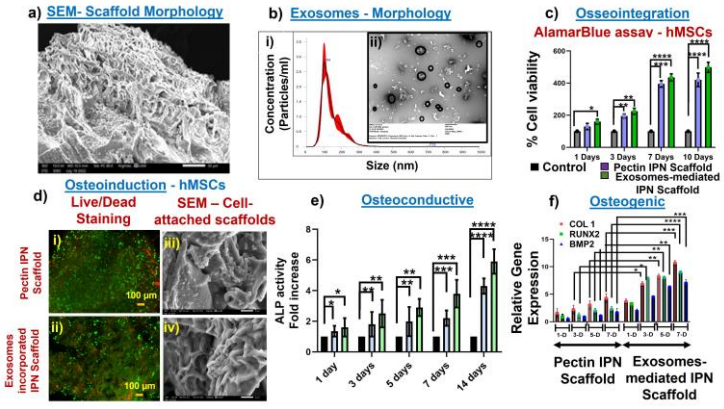

Figure 1: The major findings of the study:
(a) **SEM image** revealed the porous nature (b) **Exosomes isolated** from hMSCs characterized via (i) **DLS** demonstrating the size around 100 nm and (ii)**TEM micrographs** corroborate the DLS data (c) **Cytocompatibility assays** prove the biocompatibility and supports proliferation of cells in the scaffolds (d-i&ii) **Live/Dead staining** corroborates the AlamarBlue assay (d-iii & iv) **SEM imaging** confirms the osseointegration property of the scaffolds (e) **ALP assay** confirms the biomineralization of the scaffolds (f) **Gene expression** normalized to the control and GAPDH (housekeeping gene) of the major bone marker genes confirming the scaffold's support in bone regeneration

SIGNIFICANCE: The developed exosome-mediated pectin-based interpenetrating polymer network (IPN) scaffolds exhibit significant promise in the realm of biomedical applications, especially in the field of bone tissue engineering. These recently developed bioactive ternary interpenetrating polymer network (IPN) scaffolds provide an intriguing and novel alternative as a substitute for traditional bone transplants, successfully overcoming their inherent constraints. The current finding possesses the potential to greatly augment the process of bone defect healing and regeneration. Moreover, these scaffolds have the potential to function as precise carriers for the delivery of certain drugs, enabling customized therapies for bone-related disorders and regeneration endeavors.

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