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Extracellular vesicles derived from bone marrow mesenchymal stem cells as pro-osteogenic agents: elucidating the role of mitochondrial metabolism in bone regeneration

Anand Ganga G[1], Gopala S[2], Urulangodi M[2], Komath M[1]

[1]Dept of Biomaterial Science and Technology Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST), Trivandrum, Kerala, India; [2]Dept of Biochemistry, Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST), Trivandrum, Kerala, India

gangaanandg@gmail.com

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Introduction

Bone regeneration is a major clinical problem, and there is a need for novel and innovative therapeutic approaches. Mesenchymal stem cells (MSCs) are promising but are limited by issues of cell delivery and reproducibility. Therefore, there is a focus on the secreted extracellular vesicles (EVs) of MSCs, which contain bioactive molecules that could potentially mimic the beneficial effects of MSCs (Biswas *et al.*, 2025; Anand, Komath and Urulangodi, 2026). In this study, the osteogenic properties of EVs derived from rat bone marrow-derived MSCs (rBMSC-EVs) were explored. The rBMSC-EVs promoted proliferation and osteogenic differentiation of progenitor cells in vitro, even in the absence of chemical inducers. A comparative proteomic analysis showed that proteins in the rBMSC-EVs were enriched for those involved in angiogenesis, tissue regeneration, and osteogenesis, together with factors involved in mitochondrial aerobic respiration and the electron transport chain (Takafuji *et al.*, 2020; Chakrabarty and Chandel, 2021; Smith and Eliseev, 2021). Functional studies showed that EV treatment led to a significant increase in mitochondrial respiration and biogenesis in target cells, thus providing a mechanistic explanation for the high energy demand of bone regeneration. In summary, the data provide evidence for rBMSC-EVs as a novel and promising cell-free therapeutic agent for bone regeneration, with therapeutic effects mediated by metabolic reprogramming, thus providing a framework for EV-based orthopaedic therapeutics.

Methods

Bone marrow was harvested from femurs and tibiae of 4-week-old Wistar rats under IAEC approval (SCTIMST Protocol No. SCT/IAEC/TR/02/116/AUG/2023). rBMSCs were isolated by direct adherence and cultured in DMEM-LG with 10% fetal bovine

serum (FBS) and 1% antibiotic-antimycotic at 37°C, 5% CO_2. rBMSCs (passages 2–8) were cultured for 48 h in DMEM with 10% EV-depleted FBS. Conditioned medium was pre-cleared, filtered (0.22 µm), concentrated, and ultracentrifuged at 100,000 × g for 60 min, washed in PBS, and stored at −80°C. EVs were characterized by (Transmission electron microscope) TEM, Dynamic light scattering (DLS), Nanoparticle tracking analysis (NTA), and Western blotting for EV markers (Théry *et al.*, 2018). Osteogenic differentiation was induced with Differentiation media (DM) (L-ascorbic acid, β-glycerophosphate, dexamethasone) or Complete media (CM), supplemented with rBMSC-EVs. Alizarin Red S (ARS) staining and calcium quantification were performed at 7, 14, 21, and 28 days. Osteogenic gene expression (ONC, OPN, RUNX2, BMP2, BMP4, OSX, OCN, ALP, COL1A1) was analysed by qRT-PCR. Mitochondrial respiration was measured using high-resolution respirometry (HRR) (Oroboros Oxygraph-2k) after 100 µg/mL FBS-EVs or rBMSC-EVs for 12–60 h. Mitochondrial DNA (mtDNA) copy number was measured by qPCR (RNR1 normalized to B2M), and mitochondrial membrane potential by tetramethylrhodamine methyl ester (TMRM) staining. For proteomic analysis, rBMSC-EVs were compared with FBS-EVs. EV proteins were lysed, digested, desalted, and analysed by LC-MS/MS (Orbitrap Eclipse). Label-free quantification used Proteome Discoverer 3.0, with statistical analysis in Metaboanalyst 6.0. Datasets were mapped against ExoCarta and MitoCarta, and functional annotation used GO/KEGG, STRING, and SR plot tools. An osteoarthritic model was induced in rats. Animals were randomly divided into: (i) sham control, (ii) OA control, (iii) EV monotherapy, (iv) PRP monotherapy, and (v) EV + PRP combinatorial therapy. Animals were monitored for general health, joint swelling, and mobility throughout the study. Behavioural assessments were performed before and after treatment to evaluate pain and locomotor function. At study endpoint, animals were euthanized, and joint tissues (cartilage, subchondral bone, synovium) were harvested for downstream assays. Results are presented as mean ± standard deviation (SD). Statistical analysis was carried out using Prism 8.0.1 (GraphPad). Figures were prepared using Adobe Illustrator 2025. Following hypothesis testing and mean comparisons appropriate to the experimental design, significance was set at $p < 0.05$. The proteomic data were analysed using a multi-step bioinformatics approach, beginning with normalization followed by significance testing using a fold-change threshold greater than 2 and an adjusted p-value below 0.05.

Results and Discussion

This study characterizes rat bone marrow mesenchymal stem cells (rBMSCs) and their extracellular vesicles (rBMSC-EVs), showing that the isolated cells express typical MSC markers ($CD90^+$, $CD105^+$, $CD34^-$) and can differentiate into adipocytes, osteocytes, and chondrocytes. EVs isolated by ultracentrifugation had a typical size (~155 nm), morphology, and EV markers (Alix, CD9, HSP70, TSG101, HSP90). Functionally, rBMSC-EVs promoted rBMSC proliferation and enhanced osteogenic differentiation in a concentration- and time-dependent manner, increasing

mineralization and upregulating osteogenic markers such as RUNX2, OSX, BMP2, BMP4, COL1A1, ALP, OCN, and OPN, even in the absence of chemical osteogenic inducers; these effects were stronger than those of FBS-EVs. Proteomic analysis revealed that rBMSC-EVs carry a distinct cargo enriched in energy metabolism, osteogenesis, extracellular matrix, and signaling proteins, including 154 mitochondrial proteins and components of the electron transport chain. Functionally, rBMSC-EVs increased oxygen consumption, maximal and ATP-linked respiration, mitochondrial DNA copy number, and mitochondrial membrane potential in recipient cells, indicating enhanced oxidative phosphorylation and mitochondrial biogenesis. MIA-induced OA caused significant gait impairments across all parameters. EV or PRP monotherapy yielded only partial functional recovery, whereas their combination restored near-normal gait, indicating a synergistic effect. Overall, the study concludes that rBMSC-EVs promote bone formation by delivering a unique mitochondrial-rich protein cargo that metabolically primes target MSCs toward osteogenesis, supporting their potential as cell-free therapeutic agents for bone regeneration.

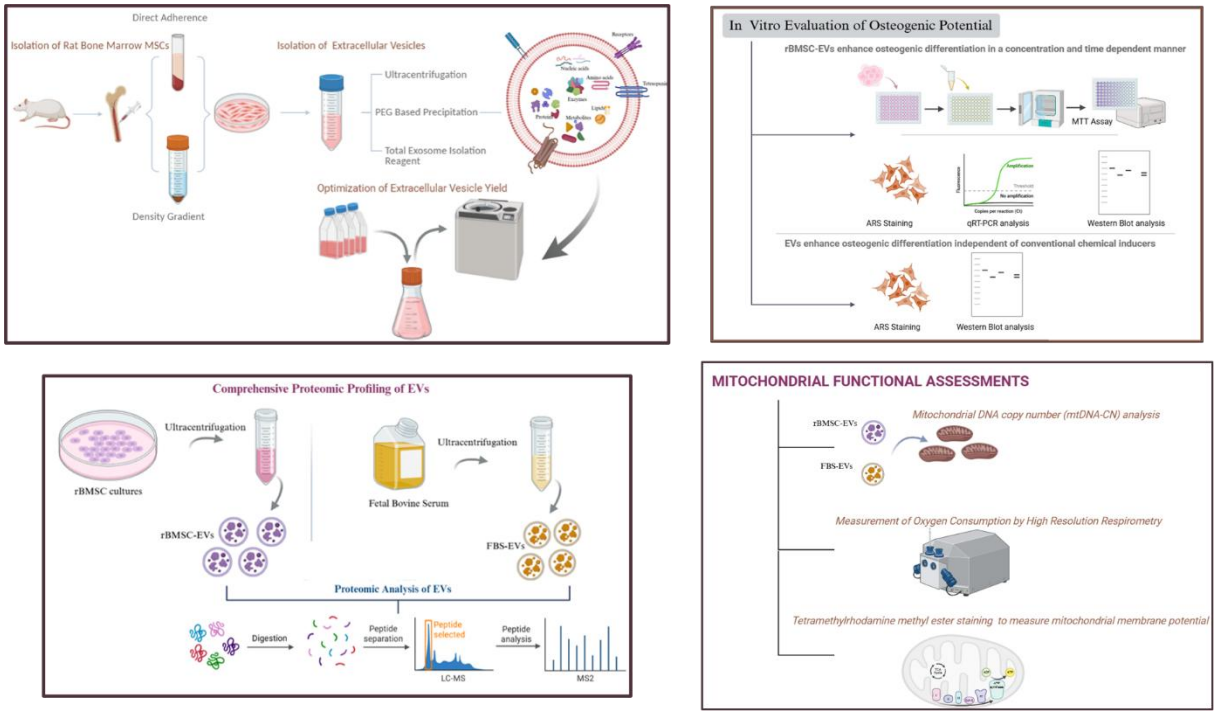

Figure 1. Summary of the workflow and methodological approaches applied in this study.

Keywords: Extracellular Vesicles, Rat Bone Marrow Mesenchymal Stem Cells, Bone Regeneration, Mitochondrial metabolism, Osteogenesis, Electron transport chain

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Conflicts of Interest: The authors declare no conflict of interest. An earlier version of this manuscript was posted as a preprint (Anand *et al.*, 2025).

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