Mesenchymal Stem Cell Extracellular Vesicles for Muscle Recovery Following Trauma

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Introduction: A substantial loss of muscle tissue (>20%), termed as a volumetric muscle loss (VML) impairs the ability of skeletal muscle to repair and regenerate itself and leads to persistent inflammation and fibrosis at the injury site. Currently, there is no definitive therapy for VML that can regenerate the lost contractile tissue or restore muscle strength. Mesenchymal stem cells (MSCs) are adult multipotent stem cells that form muscle tissue under specific conditions. Besides muscle tissue formation, MSCs support blood vessel growth, release various growth factors, suppress inflammation, and prevent fibrosis. Studies show that MSCs typically don't survive after transplantation. However, the therapeutic benefits of MSCs may still be realized by relying on their secreted products that are packaged in extracellular vesicles (EVs). These nanovesicles (30-100 nm) contain bioactive molecules such as lipids, proteins, mRNAs, and microRNAs (miRNAs) unique to the cell of origin. Studies have shown that MSC-derived EVs can polarize macrophages towards the anti-inflammatory M2 phenotype, accelerate wound healing, and increase tissue regeneration. Furthermore, exposing cells to a controlled low oxygen environment (or hypoxia) can promote stem cell survival post-transplantation and angiogenesis. We hypothesized that EVs derived from hypoxic and normoxic MSC cultures would have different biological effects pertaining to immunomodulation and tissue regeneration.

Methods: EVs were isolated from cell-culture supernatants of murine bone-marrow derived MSCs that were cultured under normoxic (21% O_2) or hypoxic (3% O_2) conditions for 48 hours at 37°C. EVs were characterized for size using Nanoparticle Tracking Analysis (NTA). Macrophages were stimulated with lipopolysaccharide (LPS) at 100 ng/ml for 24 hours in a 48 well plate at a density of 300,000 cells/well (n=3 per group). After 24 hours, LPS stimulated macrophages were washed and exposed to fresh media (control group), or media containing 25µg/mL of either normoxia or hypoxia EVs. After 72 hours of culture under standard conditions (37°C with 5% CO_2), cell-culture supernatants were harvested and stored at -20ºC until needed for ELISA tests.
EVs derived from hypoxic or normoxic MSC cultures were encapsulated into fibrin gels at a concentration of 4.48×10^{10} particles/ml and implanted into a ~20% VML defect into both gastrocnemius muscles of 129S1/SumJ mice. Plain fibrin gels were used in the control group (n=3-4/group). The mice were allowed to recover for 14 days, and muscles were harvested for histological and biochemical analysis. The procedures were approved by the Saint Louis University Institutional Animal Care and Use Committee (AUP#2612). Results were analyzed using one-way ANOVA.

Results: NTA showed that EVs derived from hypoxia-cultured MSCs have a greater concentration of particles in the 30-150 nm size range than those derived from normoxia-cultured MSCs. Macrophages exposed to normoxia and hypoxia MSC-derived EVs showed differences in secretion of trophic factors. Tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) are both classified as pro-inflammatory or M1-phenotype associated cytokines. The release of TNF-α was suppressed by EVs derived from both normoxic (p=0.0248) and hypoxic (p=0.0093) MSC cultures (Figure 1a), whereas the release of IL-6 was only inhibited by hypoxic EVs (p=0.0358) (Figure 1b). Vascular endothelial growth factor (VEGF) promotes angiogenesis and regeneration and is associated with the M2 phenotype. The release of VEGF was found to be elevated above the control group by EVs derived from both normoxic (p=0.0003) and hypoxic (0.0024) cultures. However, hypoxic EVs induced less VEGF release from macrophages compared to normoxic EVs (Figure 1c).
The EVs derived from normoxic and hypoxic MSCs were encapsulated in fibrin gels and implanted in a VML defect (Figure 2). On day 14 post-injury, treatment with gel-encapsulated normoxic-EVs resulted in higher muscle mass relative to both gel alone (p=0.0318) and gel-encapsulated hypoxic-EVs group (p=0.0098).

Discussion: Overall, these results show that hypoxia can increase the secretion of EVs from MSCs *in vitro*. Our results also indicate that hypoxia alters the composition of EVs, which can impact biological processes. Normoxic EVs enhanced the release of both IL-6 and VEGF from macrophages, suggesting activation of both pro- (M1) and anti-inflammatory (M2) phenotypes. Although lower in concentration, hypoxic EVs only enhanced VEGF release from macrophages, indicating the stimulation of an M2 phenotype. When implanted in a mouse hindlimb VML model, fibrin hydrogel encapsulated normoxic-EVs resulted in higher muscle mass relative to other groups, suggesting higher contractile tissue deposition. These results may indicate that concerted actions of both M1 and M2 macrophages are necessary for VML repair. Future studies will focus on histological and biochemical analysis of the harvested muscle samples to investigate these claims further.

Significance/Clinical Relevance: VML contributes to long-term disability in civilian and military trauma patients. Severe and persistent inflammation is a major impediment to muscle recovery post-VML. Our approach uses a cell-free strategy to support immunomodulation and muscle regeneration following VML. A significant advantage of using EVs is avoiding issues associated with stem cell transplantation, such as poor cell survival, engraftment, and potential pulmonary embolism. The therapeutic potential of MSC-derived EVs is being investigated in clinical trials for various conditions. Our results highlight that MSCs cultured under normoxic or hypoxic conditions can release EVs with different biological effects both *in vitro* and *in vivo*.

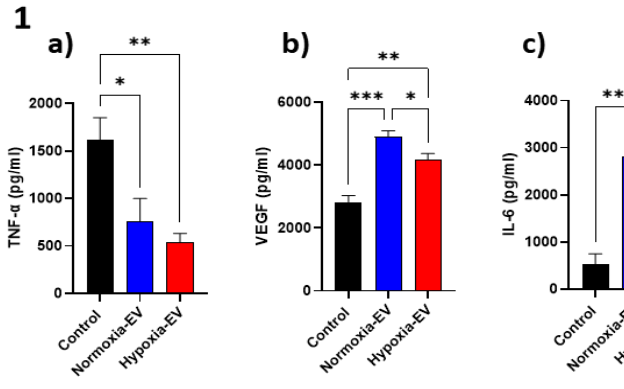

Figure 1: The concentration of a) TNF-alpha, b) IL-6, and c) VEGF in macrophage cell culture supernatants.

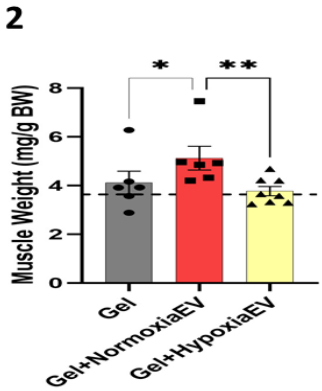

Figure 2: Muscle mass normalized to body weight for each experimental group. Gel encapsulated Normoxic-EVs enhanced muscle mass at day 14 post-VML.