Ischemic Myocardial Inflammatory Signaling in Starvation vs. Hypoxia-Derived Extracellular Vesicles: A Comparative Analysis

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Graphical Abstract.

CON MVM HYP

Macrophage Invasion

Starvation EV Treatment

NLRP3, IL-17, HLA-DRA

Overall Inflammation

Hypoxia EV Treatment

NLRP3, IL-17, IL-6, Pro-IL1β

CON MVM HYP
Title: Ischemic Myocardial Inflammatory Signaling in Starvation vs. Hypoxia-Derived Extracellular Vesicles: A Comparative Analysis

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Conflict of Interest:
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The Institutional Review Board (IRB) or equivalent ethics committee of the Warren Alpert Medical School of Brown University and Rhode Island Hospital approved the study protocol and publication of data. The patient(s) provided informed written consent for the publication of the study data.
Perspective Statement:
EV-based therapies show promise in reducing inflammation and improving outcomes in chronic ischemic heart disease. To advance the field and improve patient care, we need to better understand the impact of EV-mediated anti-inflammatory effects on the quality of life of individuals with coronary heart disease.

Central message:
Intramyocardial injection of hypoxia-preconditioned human bone mesenchymal stem cell-derived extracellular vesicles shows greater anti-inflammatory effect compared to starvation-derived vesicles.

Central picture: "Schematic Overview of Intramyocardial Injection Comparison: Starvation-Derived vs. Hypoxia-Derived Extracellular Vesicles in Swine with Induced Chronic Myocardial Ischemia" This central picture is a visualization of the study's comparison between intramyocardial injection of starvation-derived extracellular vesicles and hypoxia-derived extracellular vesicles in swine with induced chronic myocardial ischemia. The diagram demonstrates that hypoxia-derived extracellular vesicles exhibit greater inhibitory effect in inflammatory signaling within the myocardium.

Keywords:
Hypoxia; Extracellular Vesicles; Myocardial Ischemia; Swine; Ameroid; Myocardial Inflammation; Chronic Myocardial Ischemia; Chronic Coronary Artery Disease.
Abbreviations:

Pro-IL-1β - Interleukin-1 beta
IL-6 - Interleukin-6
IL-10 - Interleukin-10
IL-12 - Interleukin-12
IL-17 - Interleukin-17
NLRP3 - "NOD-like" receptor (NLR) proteins
HLA-DRA - HLA class II histocompatibility antigen
TNFα - Tumor Necrosis Factor alpha
IFN-γ - Interferon gamma
TLR2 - Toll-like receptor 2
NF-κB - Nuclear factor kappa-light-chain-enhancer of activated B cells
ABSTRACT

Objective: Coronary artery disease remains a leading cause of death worldwide. Bone mesenchymal stem cell-derived extracellular vesicles (EVs) have shown promise in the setting of myocardial ischemia. Furthermore, the properties of the EVs can be modified via pre-conditioning of the progenitor cells. Previous research from our lab demonstrated a significant decrease in pro-inflammatory signaling following treatment with EVs derived from starvation preconditioning of human bone mesenchymal stem cells (MVM EVs) in a porcine model of chronic myocardial ischemia. However, rodent models have demonstrated that the use of EVs derived from hypoxia preconditioning of bone mesenchymal stem cells (HYP EVs) may have extended benefits compared to that of MVM EVs. The purpose of this study is to evaluate the effect of HYP EVs on inflammation in a swine model of chronic myocardial ischemia. We hypothesize that HYP EVs will have a greater anti-inflammatory effect than MVM EVs or saline (CON).

Methods: Yorkshire swine fed a standard diet underwent placement of an ameroid constrictor to the left circumflex artery. Two weeks later, the animals received intramyocardial injection of saline (CON; n=6), starvation-derived EVs (MVM; n=10), or hypoxia-derived EVs (HYP; n=7). After five weeks, myocardial perfusion was assessed and left ventricular myocardial tissue was harvested. Protein expression was measured using immunoblotting. Data was analyzed via Kruskal-Wallis test or One-way ANOVA based on the results of a Shapiro–Wilk test. The coronary perfusion was plotted against relative cytokine concentration and analyzed with Spearman's rank test.
**Results:** HYP EV treatment was associated with decreased expression of pro-inflammatory markers IL-6 (p=0.03), Pro-IL1β (p=0.01), IL-17 (p<0.01) and NLRP3 (p<0.01) compared to CON. Ischemic tissue from the MVM group showed significantly decreased expression of pro-inflammatory markers NLRP3 (p<0.01), IL-17 (p<0.01), HLA-DRA (p<0.01) compared to CON. The MVM group also had decreased expression of anti-inflammatory IL-10 (p=0.01) compared to CON counterparts. There were no significant differences in expression of TNFα, IFN-γ, IL-12, TLR2, NFκβ in either group (Table 1). There was no correlation between coronary perfusion and cytokine concentration in the MVM or HYP groups, at rest or with pacing.

**Conclusions:** HYP EVs and MVM EVs appear to result in relative decreases in the degree of inflammation in chronically ischemic swine myocardium, independent of coronary perfusion. It is possible that this observed decrease may partially explain myocardial benefits seen with both HYP and MVM EV treatment.
INTRODUCTION

End-stage coronary artery disease (CAD) remains the leading cause of death worldwide, highlighting the pressing need for effective therapeutic strategies (1). The limited efficacy of standard medical and surgical approaches necessitates the exploration of alternative interventions to address this significant clinical challenge (2).

Mesenchymal stem cell-derived extracellular vesicles (EVs) have emerged as a potential therapeutic strategy that holds promise due to their ability to transport diverse biologically active molecules, including proteins, cytokines, lipids, and nucleic acids, between cells (3,4). These membrane-bound structures play a vital role in intercellular communication and molecular pathway regulation (4,5). Studies utilizing small-animal models, particularly rats and mice, have demonstrated improved cardiac function and modulation of cardiac inflammation with EV administration (6).

Previous investigations employing a swine model of chronic myocardial ischemia have shown intramyocardial injection of EVs derived from normoxia serum-starved human bone marrow mesenchymal stem cells (HBMSCs) may be beneficial with findings showing enhanced cardiac function and reduced inflammation (7,8). Animal studies have further elucidated the multifaceted roles of EVs in cardiac function, angiogenesis, cardiac regeneration, inflammation, and myocardial remodeling (7-9).

Importantly, the contents of EVs can be influenced by the conditions to which the progenitor HBMSCs are exposed (10). Specifically, hypoxia preconditioning of MSCs has been shown to enrich EV contents with proangiogenic growth factors and microRNAs compared to normoxic conditions (10). Investigations in small-animal models of acute myocardial infarction
have demonstrated potential cardioprotective effects of exosomes derived from hypoxia-
preconditioned MSCs in comparison to those derived from normoxia-preconditioned MSCs (11).

Despite evidence from studies conducted in small-animal models of acute myocardial
infarction, which have demonstrated the promising effects of HYP EVs in reducing infarct size
and overall apoptosis, there remains a considerable knowledge gap that necessitates further
scientific exploration (12). Specifically, there is a need to comprehensively investigate the
therapeutic potential of HYP EVs in the context of myocardial ischemia, particularly when
utilizing clinically relevant large-animal models.

In our previous investigation utilizing a large animal model, our group successfully
demonstrated that HYP EVs exhibit enhanced contractility, capillary density, and angiogenic
signaling pathways compared to MVM EVs (13). These findings suggest significant potential for
HYP EVs in improving cardiac function compared to MVM EVs (13). However, the underlying
mechanism remains unclear. To further understand the cardiac benefits of EVs, we plan to compare
the anti-inflammatory effects of MVM EVs and HYP EVs in a swine model of chronic myocardial
ischemia. We anticipate that HYP EVs will demonstrate stronger therapeutic potential in
mitigating myocardial inflammation.

METHODS

EV Culture and Isolation:

Human bone marrow-derived stem cells (HBMSC) from Lonza (Allendale, NJ, USA) were
cultured in accordance with Lonza recommendations using growth media (MSCGM Bulletkit PT-
3001; Lonza, Allendale, NJ, USA), as described previously (13). HYP EVs were grown until they
reached 80% confluence and then passaged to passage 7. At this stage, the media was replaced
with MSCGM media, and the cells were placed in a humidified hypoxia chamber (Billups-Rothenberg, MIC-101, Del Mar, CA, USA) containing 5% carbon dioxide and 95% nitrogen (13). The cells were incubated at 37°C for 24 hours, at which point the media and the EVs were collected as previously described via ultracentrifugation (13). Protein quantification was performed using a radioimmunoprecipitation assay (Kit 23225, Thermo Fisher Scientific, Waltham, MA, USA). Characterization was determined through immunoblotting, nanoparticle tracking analysis, and transmission electron microscopy (13). 50 ug of EVs were thawed and resuspended in 2mL of 0.9% sterile saline on the day of administration.

Large-animal Model:

Twenty-three Yorkshire swine from Cummings School of Veterinary Medicine at Tufts University Farm in North Grafton, MA, USA, were included in this study. The Institutional Review Board (IRB) or equivalent ethics committee of the Warren Alpert Medical School of Brown University and Rhode Island Hospital approved the study protocol and publication of data (10/12/2021, 1791190.). The patient(s) provided informed written consent for the publication of the study data. The animals underwent a left thoracotomy with placement of an ameroid constrictor (Research Instruments SW, Escondido, CA, USA) on the left circumflex artery (LCx) to induce chronic myocardial ischemia.

After a two-week recovery period, the swine underwent a redo left thoracotomy procedure and were divided into three groups based on the intramyocardial injection they received: normal saline (CON; n=6), starvation-derived EVs (MVMs; n=10), or hypoxia-modified EVs (HYPs; n=7). Post-injection, the swine were closely monitored for five weeks. At the conclusion of the observation period, the animals were euthanized, and tissue samples were collected for further
analysis (Figure 1). All experiments were approved by the Institutional Animal Care and Use Committee of the Rhode Island Hospital, and animals were cared for in coordination with veterinary technicians at Rhode Island Hospital in compliance with the *Principles of Laboratory Animal Care* formulated by the National Society of Medical Research and the *Guide for the Care and Use of Laboratory Animals*.

**Ameroid Constrictor placement procedure:**

Anesthesia and preoperative care were administered as previously described (13). A left thoracotomy was performed in the second intercostal space using the same technique as previously reported (13). The pericardium was opened to expose the left circumflex artery (LCx), upon which the swine received intravenous heparin at a dose of 80 IU/kg, and a vessel loop was placed around the LCx for 2 minutes of occlusion. To map the area of ischemia, five milliliters of gold microspheres (BioPal, Worcester, MA) were injected into the left atrium during the LCx occlusion which was confirmed by ST and/or T wave changes on ECG (13). The vessel loop was relaxed and a titanium-rim ameroid constrictor was placed and locked around the LCx to induce chronic myocardial ischemia over 2 to 3 weeks. Nitroglycerin was administered over the vessel as needed to reverse vasospasm. The pericardium was filled with 5 ml of normal saline to limit adhesions and closed using absorbable suture, and the layers of the chest were closed as previously described, and the pigs recovered from anesthesia in a monitored setting (13).

**Intramyocardial EV Injection procedure:**

Following the previously mentioned administration of anesthesia, perioperative analgesia, prophylaxis, and sterile preparation and draping, a left mini-thoracotomy incision was performed
one rib space below the previous thoracotomy incision associated with the ameroid placement procedure (13). The pericardium was opened and carefully secured using silk sutures, thereby revealing the ischemic left ventricular myocardium located beneath the previously implanted ameroid constrictor. Subsequently, depending on the assigned surgical group, the animals underwent intramyocardial injection.

In the MVM and HYP group, the injection involved administering EVs (50 µg) suspended in 2 mL of 0.9% saline, while the CON group received a 2 mL injection of 0.9% saline alone. The myocardium was injected at ten specific locations adjacent to the left circumflex (LCx) territory in all three experimental groups (13).

Finally, the pericardium was closed using absorbable sutures, and the chest was subsequently closed per the same aforementioned procedure.

**Left Ventricular myocardial tissue Harvest:**

Following five weeks of treatment, pigs underwent hemodynamic and functional studies before harvest. Vital signs and hemodynamic parameters were recorded using standard techniques (14). To preserve the tissue, anesthesia was deepened. Harvest involved an open incision and Seldinger technique for femoral artery access, through which a pressure monitor was inserted using a 6F catheter sheath. A median sternotomy was then performed to expose the heart. Isotope-labeled microspheres were injected into the left atrium for blood flow analysis, while 10mL of blood was simultaneously withdrawn from the femoral artery catheter. This procedure was repeated while pacing the heart to 150bpm. The heart was then removed, and myocardial tissue was promptly divided into 16 segments based on their location in relation to the left anterior descending and left circumflex arteries. These myocardial tissue segments were either snap frozen in liquid nitrogen...
for immunoblotting analysis, frozen sectioning or air dried for microsphere analysis (14). See Figure 4 for a graphical abstract of the study.

**Left Ventricular myocardial Perfusion:**

Myocardial perfusion was determined using isotope-labeled microspheres (Biophysics Assay Laboratory, Worcester, MA). During the ameroid placement procedure, five mL of gold microspheres were injected into the left atrial appendage while occluding the LCx with a vessel loop to map the ischemic left ventricular area supplied by the LCx. During the harvest procedure, 5mL of Lutetium-labeled microspheres were injected into the left atrium while simultaneously withdrawing 10ml of blood from the femoral artery (13). Then the heart was paced at 150bpm and 5mL of Samarium-labeled microspheres were injected into the left atrium while simultaneously withdrawing 10mL of blood from the femoral artery (13). Blood samples and left ventricular myocardial samples from 10 sections based on proximity of location to the LAD and left circumflex arteries were weighed, dried, and sent to Biophysics Assay Laboratory to measure microsphere density for blood flow analysis, allowing for blood flow analysis to be performed on ischemic tissue segments for each pig (14).

**Protein extraction and Immunoblotting:**

Ischemic myocardial tissue samples were obtained from six control animals and seventeen experimental animals and subsequently lysed using RIPA Lysis and Extraction Buffer supplemented with Halt Protease Inhibitor Cocktail (ThermoFisher Scientific, Waltham, MA, USA) and an ultrasonic homogenizer (14). Protein quantification was performed utilizing a BCA Protein Assay Kit (ThermoFisher Scientific, Waltham, MA, USA). The lysates were loaded onto
a 4-12% Bis-Tris gel (ThermoFisher Scientific, Waltham, MA, USA) for electrophoresis, and the separated proteins were subsequently transferred onto nitrocellulose membranes (ThermoFisher Scientific, Waltham, MA, USA) (14).

After blocking with 5% non-fat dry milk in TBST (Boston BioProducts, Milford, MA, USA) for one hour, the membranes were incubated overnight at 4°C with primary antibodies (14). After the membranes were washed, Horseradish peroxidase-conjugated secondary antibodies against mouse or rabbit (Cell Signaling, Danvers, MA) were prepared at a dilution of 2.5:10,000 in TBST containing 3% bovine serum albumin. The membrane was then incubated with the secondary antibodies at room temperature for one hour (14). Membranes were then washed and processed for chemiluminescent detection and captured with a digital camera system (Bio-Rad ChemiDoc MP, Life Science, Hercules, CA) (14).

The membranes were imaged on a ChemiDoc Imaging System (Bio-Rad, Hercules, CA, USA) using ECL Western Blotting Substrate (ThermoFisher Scientific, Waltham, MA, USA) as the developing agent. Membranes were stripped with Restore PLUS Western Blot Stripping Buffer (ThermoFisher Scientific, Waltham, MA, USA) to allow for repeat probing. All membranes were probed with GAPDH or α-tubulin (Cell Signaling, Danvers, MA) to correct for loading error. Densitometric analysis of band intensity was performed using NIH Image J software (14).

**Primary Antibodies:**

The primary antibodies were prepared by diluting them to a concentration of 1:1,000 in TBST (Tris-buffered saline with Tween) solution containing 3% bovine serum albumin. The specific primary antibodies used in this study included those targeting Interleukin-1 beta (Pro-IL-1β), Interleukin-6 (IL-6), Interleukin-10 (IL-10), Interleukin-12 (IL-12), Interleukin-17 (IL-17),
NOD-like receptor proteins (NLRP3), HLA class II histocompatibility antigen, DR alpha chain (HLA-DRA), Tumor Necrosis Factor alpha (TNFα), Interferon gamma (IFN-γ), Toll-like receptor 2 (TLR2), and Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). These primary antibodies were obtained from Cell Signaling (Danvers, MA, USA).

Immunohistochemistry:

The immunohistochemistry technique was conducted following the established protocol described (15). In summary, frozen section slides were thawed, fixed using 10% paraformaldehyde, blocked, and then incubated with a CD68 antibody from Cell Signaling (Danvers, MA). Images were captured at 20X magnification using a Nikon E800 Eclipse microscope (Nikon, Tokyo, Japan). Macrophages were identified based on CD68 staining and quantified for each specimen in multiple high-powered fields, with an average count calculated per specimen. Neutrophils were identified based on neutrophil elastase staining, and their counts were similarly quantified. Representative images can be found in the manuscript (Figure 3).

Statistical Analysis:

Analysis was completed using Prism 9 software (GraphPad Software, San Diego, CA, USA). The normality of the data was assessed using the Shapiro-Wilk test. For non-parametric data, the Wilcoxon rank-sum test was employed, while Student's t-test was used for the analysis of normally distributed data.

The results are presented as the mean ± standard deviation. In the case of Western blot data, the mean fold change was calculated and normalized to the average control value. Data points that deviated from the mean by more than two standard deviations were excluded from the analysis.
to ensure the reliability of the statistical results. Immunohistochemistry for macrophages was determined by CD68 staining and reported as median macrophage count/high-powered field with interquartile ranges.

Western blotting and immunohistochemistry data were statistically analyzed via Wilcoxon rank-sum test. Probability values <0.05 were considered significant.

RESULTS

Protein Expression:

In the ischemic tissue obtained from the MVM group, a significant decrease in the expression of pro-inflammatory markers was observed compared to the CON group. Specifically, the MVM group exhibited a significant reduction in the expression levels of NLRP3 (p<0.01), IL-17 (p<0.01), and HLA-DRA (p<0.01) (Table 1, Figure 2).

The MVM group had reduced expression of the anti-inflammatory marker IL-10 (p=0.01) compared to the CON group (Table 1).

HYP EV treatment resulted in a significant decrease in the expression of various pro-inflammatory markers when compared to the CON group. Specifically, the HYP group demonstrated a significant reduction in the expression levels of IL-6 (p=0.03), Pro-IL1β (p=0.01), IL-17 (p<0.01), and NLRP3 (p<0.01) (Table 1, Figure 2).

No significant differences were observed in the expression levels of TNFα, IFN-γ, IL-12, TLR2, and NFκB in either the MVM or HYP group (Table 1). Additionally, no significant correlations were found between coronary perfusion and cytokine concentration in either the MVM or HYP groups, both at rest and with pacing, mitigating the influence of myocardial perfusion on the results (Table 2).
In the non-ischemic tissue obtained from the MVM group, a significant decrease in the expression of pro-inflammatory markers was observed compared to the CON group (Figure 5). Specifically, the MVM group exhibited a significant reduction in the expression levels of NLRP3 (p<0.05), IL-17 (p<0.05), HLA-DRA (p<0.05), IL-6 (p<0.001) and Pro-IL1β (p=0.05) compared to the control group with no changes in the anti-inflammatory marker IL-10 (Figure 5). While HYP EV’s showed only a significant decrease in NLRP3 (p<0.05) compared to control in the non-ischemic tissue (Figure 5). No significant differences were observed in the expression levels of HLA-DRA, Pro-IL1β, IL-17, IL-6 and IL-10 in the HYP group compared to control (Figure 5).

Macrophage and neutrophil Counts:
In ischemic myocardium, there were no statistically significant variations in the total number of macrophages and neutrophils per high-powered field (HPF) between the groups treated with extracellular vesicles (EV) and the control group in the ischemic myocardium.

Blood Flow Correlation:
Among EV-treated pigs, there was no significant correlation between blood flow to ischemic territory and protein expression of Pro-IL-1β, IL-6, IL-10, IL-12, IL-17, NLRP3, HLA-DRA, TNFα, IFN-γ, TLR2 and NF-κB at rest and during pacing. (Table 2).

DISCUSSION
In this study, we investigated the effects of starvation- and hypoxia-derived extracellular vesicles (EVs) on inflammation in the left ventricular ischemic myocardial tissue of pigs. Our findings provide evidence that both starvation- and hypoxia-derived EVs have anti-inflammatory
effects in the ischemic myocardium, with hypoxia-derived EVs demonstrating greater potency compared to starvation EVs derived from normoxic conditions.

We observed a significant reduction in the expression levels of four pro-inflammatory markers, including IL-6, Pro-IL1β, IL-17, and NLRP3, in the myocardial tissue treated with HYP EVs. On the other hand, MVM/standard EVs showed a significant decrease in the expression levels of three pro-inflammatory markers, namely NLRP3, IL-17, and HLA-DRA. These findings suggest that HYP EVs may have a superior anti-inflammatory effect in the context of chronic myocardial ischemia compared to MVM EVs and highlight the potential of both types of EVs to mitigate inflammation in the ischemic myocardium.

Inflammatory signaling mediators, such as IL-1β and IL-6, are known to play important roles in the development of cardiac remodeling and impaired cardiac function following ischemic injury (16, 17). Consistent with our findings, in previous studies conducted in our laboratory, we observed a significant decrease in pro-IL-1β levels in the chronically ischemic myocardium of swine with metabolic syndrome after treatment with MVM EVs (8). This reduction in pro-inflammatory signaling through EV therapy could potentially explain the observed improvement in cardiac function and perfusion associated with intramyocardial EV injections (7, 14).

Moreover, in a previous study conducted in our laboratory, treatment with HYP EVs demonstrated several beneficial effects when compared to treatment with MVM EVs in a swine model of chronic myocardial ischemia (13). Specifically, treatment with HYP EVs led to a significant increase in capillary density and improved left ventricular contractility in the chronically ischemic myocardium of swine, surpassing the outcomes observed with MVM EV treatment (13).
Consistent with these findings, our current study confirms that both MVM EVs and HYP EVs effectively reduce pro-IL-1ß levels as shown in figure 2 which displays protein expression of cytokines in the chronically ischemic myocardium (Table 1, Figure 2). Additionally, HYP EVs demonstrated a significant reduction in IL-6 levels compared to MVM EVs in the same myocardial tissue, while MVM EVs had no significant effect on IL-6 levels (Table 1, Figure 2). This reduction in IL-6 mediated by HYP EVs may partially explain the observed increase in capillary density and left ventricular contractility in swine treated with HYP EVs compared to those treated with MVM EVs in the context of chronically ischemic myocardium (13).

Overall, our study highlights the superior anti-inflammatory properties of HYP EVs compared to MVM EVs (Table 1, Figure 2). The stronger anti-inflammatory effect observed with HYP EVs may partially account for the previously reported increase in myocardial capillary density and improved left ventricular contractility observed in the swine model of chronic myocardial ischemia (13). These findings suggest that the enhanced anti-inflammatory properties of HYP EVs contribute to their superior therapeutic potential in the treatment of chronic myocardial ischemia.

An important aspect of our study is the independence of the observed decrease in inflammatory markers from coronary perfusion (Table 2). We evaluated coronary perfusion using gold microspheres and found that the reduction in inflammation in the left ventricular myocardium was not attributable to improvements in blood flow (14). This suggests that the anti-inflammatory effects of EVs are mediated through mechanisms beyond enhanced perfusion (Table 2). The significant reduction in inflammation in the myocardial tissue of both the starvation- and hypoxia-derived EV groups may provide insights into the potential mechanisms underlying the observed myocardial benefits associated with EV treatment.
Chronic myocardial ischemia is characterized by ongoing inflammation, and attenuating this inflammatory response is crucial for managing the disease (18, 19). While our study sheds light on the anti-inflammatory effects of EVs, it is important to note that coronary heart disease is a complex condition influenced by various factors (20). Therefore, addressing inflammation in the myocardium typically requires a comprehensive treatment approach involving lifestyle modifications, medication, and, in some cases, invasive procedures such as angioplasty or bypass surgery (21, 22). Further research, including clinical trials, is necessary to evaluate the clinical implications, efficacy, and long-term outcomes of EV therapy in individuals with coronary heart disease.

Our study highlights the potential of EV-based therapies as a promising approach to mitigate inflammation and improve outcomes in chronic ischemic heart disease (7,13). The observed decrease in inflammation in the myocardium, independent of coronary perfusion, suggests the direct anti-inflammatory mechanisms of EVs (Table 2). Advancing the field and improving patient care require a comprehensive understanding of the potential impact of EV-mediated anti-inflammatory effects on the quality of life for those suffering from coronary heart disease (23). Additionally, targeting the dysregulation of NLRP3 inflammasome activation, implicated in various inflammatory and metabolic disorders as well as ischemia/reperfusion (I/R) injuries, holds promise as a therapeutic strategy (24). Further research in this area could provide new insights into the treatment of inflammatory and metabolic diseases associated with NLRP3 inflammasome dysregulation (24).

These results highlight the distinct effects of the MVM intervention and HYP EV treatment on the expression of pro-inflammatory and anti-inflammatory markers in the context of chronic myocardial ischemia. The reduction in pro-inflammatory markers in the MVM group and the
significant decrease in multiple pro-inflammatory markers in the HYP group indicate the potential anti-inflammatory effects of these interventions. Furthermore, we found that the HYP EVs were more effective in the ischemic tissue, in reducing pro-inflammatory markers as opposed to the non-ischemic tissue, whereas the MVM EVs were more effective in the non-ischemic tissue compared to the ischemic tissue (Figure 2, Figure 5). Further investigations are needed to elucidate the underlying mechanisms and the clinical implications of these findings which is further discussed in a study by Sabe et al (25).

We would hypothesize that the differential effects of HYP and MVM EVs on inflammatory cytokines may be related to their different cargo compositions, particularly microRNAs, which are known to regulate various aspects of inflammation. Specific microRNAs contained within the EVs may be responsible for differential regulation of some inflammatory pathways over others, though further studies by our lab into the transcriptomic profile of the EVs will provide better mechanistic clarity.

This study is subject to limitations, such as a small sample size, measurement at a single time point, and the limited clinical feasibility of intramyocardial EV delivery (13). To advance our knowledge in this field, it is important for future research to address these limitations. Nevertheless, our protocols and techniques can provide valuable guidance for future studies (13).

CONCLUSION

Our study demonstrates that both starvation- and hypoxia-preconditioned extracellular vesicles (EVs) exert a significant reduction in inflammation within the myocardium of swine with chronic ischemia (Table 1, Figure 2). This anti-inflammatory effect is independent of coronary perfusion, indicating that the observed decrease in inflammation is not solely attributed
to improved blood flow (Table 2). The findings suggest that EVs derived from hypoxic conditions (HYP EVs) may possess a superior anti-inflammatory effect compared to those derived from normoxic conditions (MVM EVs) in the context of chronic myocardial ischemia.

REFERENCES:


Visual Abstract: Comparative Analysis of Normoxia- and Hypoxia-Modified Extracellular Vesicles on Myocardial Inflammation in Chronic Coronary Artery Disease: An Investigation in a Large-Animal Model. Twenty-Three Yorkshire swine were assigned to three
groups: saline control injection (CON; n=6) or starvation-derived EVs (MVM; n=10) or hypoxia-derived extracellular vesicles (HYP; n=7). Swine underwent placement of an ameroid constrictor on the left coronary circumflex artery at age 11 weeks. Two weeks later, all swine underwent redo-left thoracotomy with injection of saline or MVM or HYP. There was no difference in the total number of macrophages per high-powered field (HPF) between both groups treated with extracellular vesicles (EV) and the control group in the ischemic myocardium. Immunoblotting showed decreased expression of pro-inflammatory proteins in both MVM and HYP groups compared to CON. Intramyocardial injection of normoxia pre-conditioned EVs resulted in decreased expression of pro-inflammatory markers NLRP3, IL-17, HLA-DRA, while intramyocardial injection of hypoxia pre-conditioned extracellular vesicle results in decreased expression of pro-inflammatory markers NLRP3, IL-17, IL-6, Pro-IL-1β. Both EVs decreased overall inflammation.

Figure 1. Schematic overview of study design. Twenty-three Yorkshire swine fed a standard diet underwent placement of an ameroid constrictor to the left circumflex artery. Two weeks later, the animals received intramyocardial injection of saline (CON, n=6), starvation-derived EVs (MVM; n=10), or hypoxia-derived EVs (HYP; n=7). After five weeks, myocardial perfusion was assessed and left ventricular myocardial tissue was harvested. Protein expression was measured using immunoblotting.

Figure 2. Relative protein expression in ischemic tissue by western blot normalized to GAPDH. Immunoblotting data is mean fold change in starvation-derived extracellular vesicle group (MVM; n =10) and hypoxia-derived extracellular vesicle group (HYP; n=7) with standard...
deviation (SD) normalized to average control (CON; n=6). Data points greater than two standard deviations from the mean are excluded from analysis. *p<0.5, **p<0.01, ***p<0.001.

Table 1. Cytokine expression in ischemic myocardium of EV-treated swine. Table shows immunoblotting results for all markers tested in the study. Immunoblotting data is mean fold change in starvation-derived extracellular vesicle group (MVM; n=10) and hypoxia-derived extracellular vesicle group (HYP; n=7) with standard deviation (SD) normalized to average control (CON; n=6). Data points greater than two standard deviations from the mean are excluded from analysis. NOD-like receptor proteins (NLRP3), Interleukin-6 (IL-6), Interleukin-17 (IL-17), Pro-Interleukin-1 beta (Pro-IL-1β), HLA class II histocompatibility antigen, DR alpha chain (HLA-DRA), Tumor Necrosis Factor alpha (TNFα), Interferon gamma (IFN-γ), Interleukin-12 (IL-12), Toll-like receptor 2 (TLR2), Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), Interleukin-10 (IL-10), Interleukin-4 (IL-4), Tumor growth factor-beta (TGF- β).

Figure 3. Macrophage invasion and neutrophil count.

There was no difference in the total number of macrophages and neutrophils per high-powered field (HPF) between both groups treated with extracellular vesicles (EV) and the control group in the ischemic myocardium.

Table 2. Correlation of coronary perfusion with cytokine concentration. There was no statistically significant correlation observed between coronary blood flow to the ischemic territory and the protein expression levels of NLRP3, IL-6, IL-17, Pro-IL-1β, HLA-DRA, TNFα,
IFN-γ, IL-12, TLR2, NFκB, p-NFκB, IL-10, IL-4, TGF-β in EV-treated pigs, both at rest and during pacing.

**Figure 4. Graphical Abstract.**

**Figure 5. Relative protein expression in non-ischemic tissue by western blot normalized to GAPDH.** Immunoblotting data is mean fold change in starvation-derived extracellular vesicle group (MVM; n =10) and hypoxia-derived extracellular vesicle group (HYP; n=7) with standard deviation (SD) normalized to average control (CON; n=6). Data points greater than two standard deviations from the mean are excluded from analysis. *p<0.5, **p<0.01, ***p<0.001.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Starvation-Derived EVs</th>
<th>Hypoxia-Derived EVs</th>
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<tr>
<td></td>
<td>Median (IQR)</td>
<td>P-value</td>
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<td>Pro-inflammatory</td>
<td></td>
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<tr>
<td>NLRP3</td>
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<tr>
<td>IL-6</td>
<td>0.84 (0.78-1.01)</td>
<td>0.44</td>
</tr>
<tr>
<td>IL-17</td>
<td>0.46 (0.35-0.60)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Pro-IL1β</td>
<td>1.05 (0.99-1.15)</td>
<td>0.77</td>
</tr>
<tr>
<td>HLA-DRA</td>
<td>0.52 (0.40-0.59)</td>
<td>0.002</td>
</tr>
<tr>
<td>TNFα</td>
<td>0.97 (0.57-2.39)</td>
<td>0.99</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>0.83 (0.75-0.92)</td>
<td>0.58</td>
</tr>
<tr>
<td>IL-12</td>
<td>0.98 (0.93-1.02)</td>
<td>0.88</td>
</tr>
<tr>
<td>TLR2</td>
<td>0.53 (0.24-0.73)</td>
<td>0.18</td>
</tr>
<tr>
<td>NFκβ</td>
<td>1.07 (1.01-1.17)</td>
<td>0.44</td>
</tr>
<tr>
<td>p-NFκβ</td>
<td>1.11 (0.90-1.40)</td>
<td>0.66</td>
</tr>
<tr>
<td>Anti-inflammatory</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>0.49 (0.38-0.62)</td>
<td>0.01</td>
</tr>
<tr>
<td>IL-4</td>
<td>1.05 (0.82-1.71)</td>
<td>0.99</td>
</tr>
<tr>
<td>TGF-β</td>
<td>0.79 (0.69-0.83)</td>
<td>0.07</td>
</tr>
</tbody>
</table>

Table 1: Cytokine expression in Ischemic myocardium of EV-treated swine
<table>
<thead>
<tr>
<th>Protein</th>
<th>At Rest R-value</th>
<th>At Rest P-value</th>
<th>With Pacing R-value</th>
<th>With Pacing P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pro-inflammatory</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NLRP3</td>
<td>&lt;0.001</td>
<td>0.92</td>
<td>0.02</td>
<td>0.46</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.002</td>
<td>0.83</td>
<td>0.04</td>
<td>0.34</td>
</tr>
<tr>
<td>IL-17</td>
<td>0.16</td>
<td>0.05</td>
<td>0.07</td>
<td>0.20</td>
</tr>
<tr>
<td>Pro-IL1β</td>
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<td>0.03</td>
<td>0.08</td>
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</tr>
<tr>
<td>HLA-DRA</td>
<td>0.001</td>
<td>0.85</td>
<td>0.005</td>
<td>0.73</td>
</tr>
<tr>
<td>TNFα</td>
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<td>0.34</td>
<td>0.14</td>
<td>0.07</td>
</tr>
<tr>
<td>IFN-γ</td>
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<td>0.10</td>
<td>0.007</td>
<td>0.69</td>
</tr>
<tr>
<td>IL-12</td>
<td>0.02</td>
<td>0.46</td>
<td>0.015</td>
<td>0.57</td>
</tr>
<tr>
<td>TLR2</td>
<td>&lt;0.001</td>
<td>0.97</td>
<td>0.005</td>
<td>0.86</td>
</tr>
<tr>
<td>NFκβ</td>
<td>0.005</td>
<td>0.75</td>
<td>0.01</td>
<td>0.63</td>
</tr>
<tr>
<td>p-NFκβ</td>
<td>&lt;0.001</td>
<td>0.96</td>
<td>0.1</td>
<td>0.12</td>
</tr>
<tr>
<td><strong>Anti-inflammatory</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>0.13</td>
<td>0.09</td>
<td>0.07</td>
<td>0.19</td>
</tr>
<tr>
<td>IL-4</td>
<td>0.01</td>
<td>0.63</td>
<td>0.17</td>
<td>0.04</td>
</tr>
<tr>
<td>TGF- β</td>
<td>0.009</td>
<td>0.66</td>
<td>0.005</td>
<td>0.74</td>
</tr>
</tbody>
</table>

Table 2: Correlation of coronary perfusion with cytokine concentration
Figure 1. Schematic overview of study design
Figure 2. Relative protein expression in ischemic tissue by western blot normalized to GAPDH.
Figure 3. Macrophage invasion and neutrophil count
**Graphical Abstract.**
Figure 5. Relative protein expression in non-ischemic tissue by western blot normalized to GAPDH.
Central picture.