Ischemic myocardial inflammatory signaling in starvation versus hypoxia-derived extracellular vesicles: A comparative analysis

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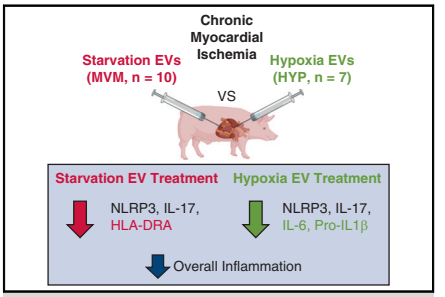
ABSTRACT

Background: 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 preconditioning of progenitor cells. Previous research from our lab demonstrated a significant decrease in proinflammatory 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 MVM EVs. This study evaluated the effect of HYP EVs on inflammation in a swine model of chronic myocardial ischemia. We hypothesized that HYP EVs would 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 5 weeks, myocardial perfusion was assessed, and left ventricular myocardial tissue was harvested. Protein expression was measured using immunoblotting. Data were analyzed via the Kruskal-Wallis test or one-way analysis of variance based on the results of a Shapiro-Wilk test. Coronary perfusion was plotted against relative cytokine concentration and analyzed with the Spearman rank-sum test.

Results: HYP EV treatment was associated with decreased expression of proinflammatory markers interleukin (IL)-6 ($P = .03$), Pro-IL-1û ($P = .01$), IL-17 ($P < .01$), and NOD-like receptor protein 3 (NLRP3; $P < .01$) compared to CON. Ischemic tissue from the MVM group showed significantly decreased expression of pro-inflammatory markers NLRP3 ($P < .01$), IL-17 ($P < .01$), and HLA class II histocompatibility antigen ($P < .01$) compared to CON. The MVM group also had decreased expression of anti-inflammatory IL-10 ($P = .01$) compared to CON counterparts. There were no significant differences in expression of tumor necrosis factor-α, interferon-γ, IL-12, Toll-like receptor-2, and nuclear factor kappa-light-chain-enhancer of activated B cells in either group . There was no correlation between coronary perfusion and cytokine concentration in the MVM or HYP groups, either 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 the myocardial benefits seen with both HYP and MVM EV treatment. (JTCVS Open 2023;16:419-28)



Visualization of the comparison between intramyocardial injection of starvation-derived EVs and hypoxia-derived EVs in swine with induced chronic myocardial ischemia. The diagram shows that hypoxia-derived EVs exhibit a greater inhibitory effect on inflammatory signaling within the myocardium.

CENTRAL MESSAGE

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

PERSPECTIVE

Extracellular vesicle (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.

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Abbreviations and Acronyms

HLA-DRA = HLA class II histocompatibility antigen
IL = interleukin
IFN = interferon
NF-κB = nuclear factor kappa-light-chain-enhancer of activated B cells
NLRP3 = NOD-like receptor protein 3
TLR2 = Toll-like receptor 2
TNF = tumor necrosis factor

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 owing to their ability to transport diverse biologically active molecules, including proteins, cytokines, lipids, and nucleic acids, between cells.[3,4] These membrane-bound structures have vital roles in intercellular communication and molecular pathway regulation.[4,5] Studies using small-animal models, particularly rats and mice, have demonstrated improved cardiac function and modulation of cardiac inflammation with EV administration.[6]

Previous investigations using a swine model of chronic myocardial ischemia suggest that 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 the 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 compared 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 using clinically relevant large-animal models.

In our previous investigation using 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

The Institutional Review Board 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. 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*.

EV Culture and Isolation

HBMSCs (Lonza) were cultured in accordance with the manufacturer's recommendations using growth medium (MSCGM Bulletkit PT-3001; Lonza), as described previously.[13] HYP EVs were grown until they reached 80% confluence and then passaged to passage 7. At this stage, the medium was replaced with MSCGM medium, and the cells were placed in a humidified hypoxia chamber (MIC-101; Billups-Rothenberg) containing 5% carbon dioxide and 95% nitrogen.[13] The cells were incubated at 37 °C for 24 hours, after which the medium and the EVs were collected via ultracentrifugation as described previously.[13] Protein quantification was performed using a radioimmunoprecipitation assay (Kit 23225; Thermo Fisher Scientific). Characterization was determined through immunoblotting,

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.

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nanoparticle tracking analysis, and transmission electron microscopy.[13] EVs (50 μg) were thawed and resuspended in 2 mL 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 were included in this study. The animals underwent a left thoracotomy with placement of an ameroid constrictor (Research Instruments SW) on the left circumflex artery (LCx) to induce chronic myocardial ischemia.

After a 2-week recovery period, the swine underwent a redo left thoracotomy procedure and were divided into 3 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). Postinjection, the swine were closely monitored for 5 weeks. At the conclusion of the observation period, the animals were euthanized, and tissue samples were collected for further analysis (Figure 1).

Ameroid Constrictor Placement Procedure

Anesthesia and preoperative care were administered as described previously.[13] A left 1thoracotomy was performed in the second intercostal space using a previously reported technique.[13] The pericardium was opened to expose the LCx, after which the swine received i.v. 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, 5 mL of gold microspheres (BioPal) were injected into the left atrium during the LCx occlusion, which was confirmed by ST and/or T wave changes on electrocardiography.[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 described previously,[13] and the pigs recovered from anesthesia in a monitored setting.

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 groups, the injection involved administering 50 μg of EVs suspended in 2 mL of 0.9% saline, whereas the CON group received a 2-mL injection of 0.9% saline alone. The myocardium was injected at 10 specific locations adjacent to the LCx territory in all 3 experimental groups.[13]

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

Left Ventricular Myocardial Tissue Harvest

After 5 weeks of treatment, the pigs were subjected to 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 10 mL of blood was simultaneously withdrawn from the femoral artery catheter. This procedure was repeated while pacing the heart to 150 bpm.

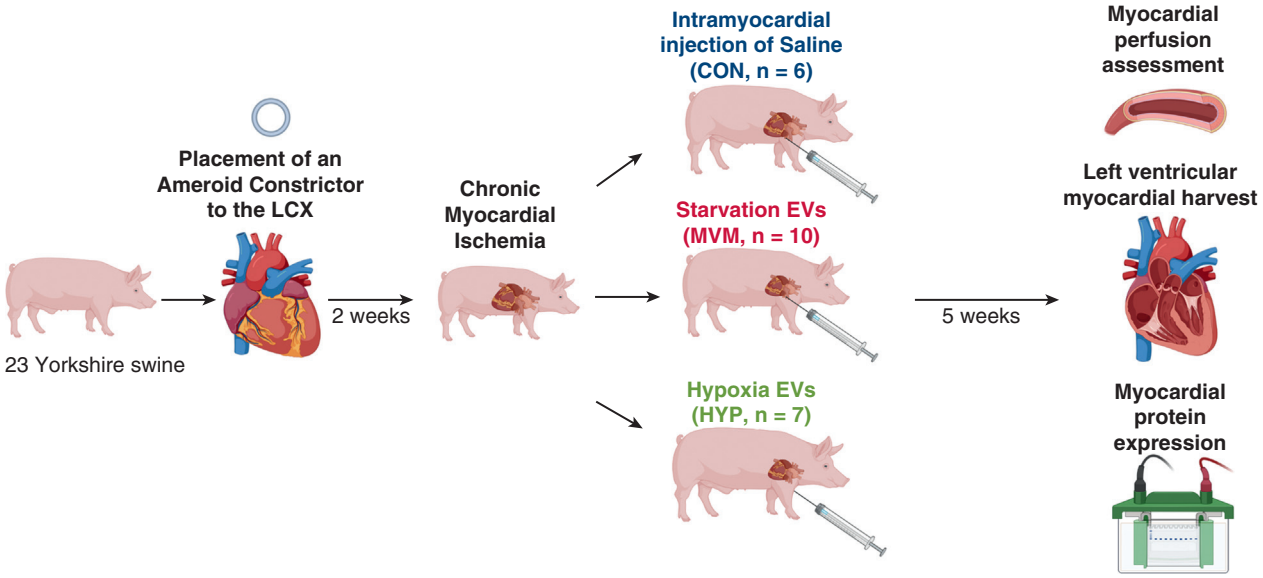

FIGURE 1. Schematic overview of study design. Twenty-three Yorkshire swine fed a standard diet underwent placement of an ameroid constrictor to the left circumflex artery (*LCx*). Two weeks later, the animals received an intramyocardial injection of saline (CON; n = 6), starvation-derived extracellular vesicles (*EVs*) (MVM; n = 10) or hypoxia-derived EVs (HYP; n = 7). After 5 weeks, myocardial perfusion was assessed, and left ventricular myocardial tissue was harvested. Protein expression was measured by immunoblotting. *CON*, Saline control group; *MVM*, starvation-derived extracellular vesicle group; *HYP*, hypoxia-derived EV group.

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 artery and LCx arteries. These myocardial tissue segments were either snap-frozen in liquid nitrogen for immunoblotting analysis of frozen sections or air-dried for microsphere analysis.[14] Figure 2 provides a graphical abstract of the study.

Left Ventricular Myocardial Perfusion

Myocardial perfusion was determined using isotope-labeled microspheres (Biophysics Assay Laboratory). During the ameroid placement procedure, 5 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, 5 mL of lutetium-labeled microspheres were injected into the left atrium while simultaneously withdrawing 10 mL of blood from the femoral artery.[13] Then the heart was paced at 150 bpm, and 5 mL of samarium-labeled microspheres were injected into the left atrium while simultaneously withdrawing 10 mL of blood from the femoral artery.[13] Blood samples and left ventricular myocardial samples from 10 sections based on proximity of location to the left anterior descending artery and LCx were weighed, dried, and sent to the Biophysics Assay Laboratory to measure microsphere density for blood flow analysis, allowing for blood flow analysis on ischemic tissue segments for each pig.[14]

Protein Extraction and Immunoblotting

Ischemic myocardial tissue samples were obtained from 6 control animals and 17 experimental animals and subsequently lysed using RIPA Lysis and Extraction Buffer supplemented with Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific) and an ultrasonic homogenizer.[14] Protein quantification was performed using a BCA Protein Assay Kit (Thermo Fisher Scientific). The lysates were loaded onto a 4% to 12% Bis-Tris gel (Thermo Fisher Scientific) for electrophoresis, and the separated proteins were subsequently transferred onto nitrocellulose membranes (Thermo Fisher Scientific).[14]

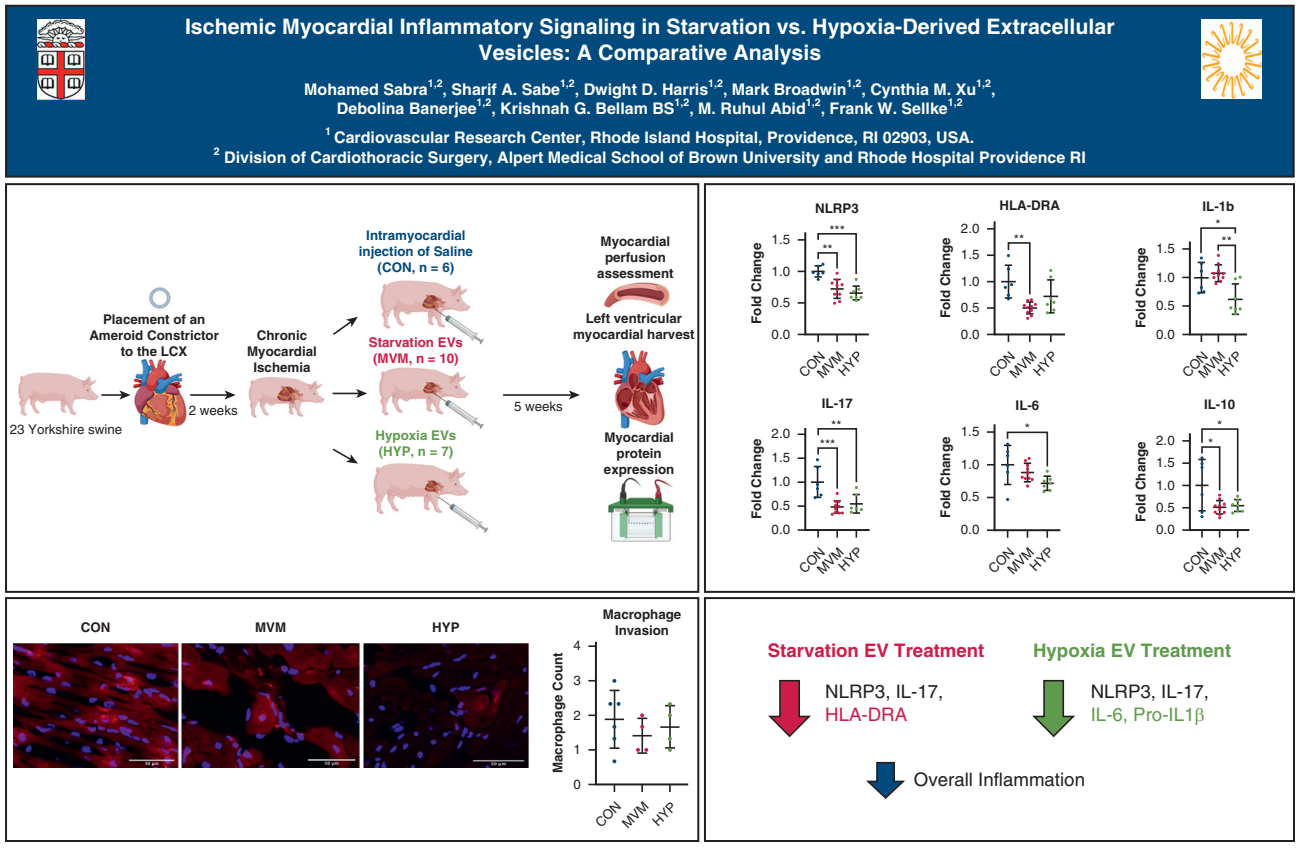

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FIGURE 2. Twenty-three Yorkshire swine were assigned to 1 of 3 groups: saline control injection (*CON*; n = 6), starvation-derived extracellular vesicles (*EVs*) (*MVM*; n = 10), or hypoxia-derived EVs (*HYP*; n = 7). Swine underwent placement of an ameroid constrictor on the left coronary circumflex (*LCx*) artery at age 11 weeks. Two weeks later, all swine underwent redo left thoracotomy with injection of saline, MVM, or HYP. There was no difference in the total number of macrophages per high-power field between both groups treated with EVs and the CON group in the ischemic myocardium. Immunoblotting showed decreased expression of proinflammatory proteins in both the MVM and HYP groups compared to CON. Intramyocardial injection of normoxia preconditioned EVs resulted in decreased expression of proinflammatory markers NOD-like receptor protein (*3NLRP3*), interleukin (*IL*)-17, and HLA class II histocompatibility antigen (*HLA-DRA*), whereas intramyocardial injection of hypoxia preconditioned EVs resulted in decreased expression of proinflammatory markers NLRP3, IL-17, IL-6, and Pro-IL-1β. Both EVs decreased overall inflammation.

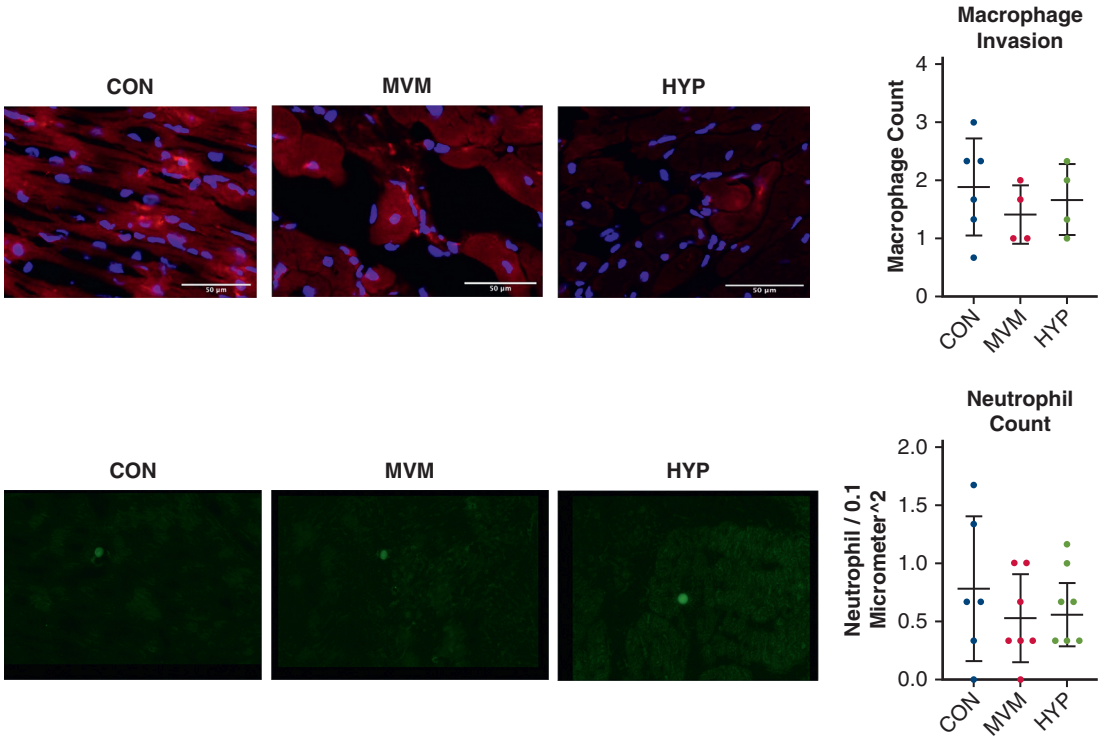

FIGURE 3. Macrophage invasion and neutrophil count in the ischemic myocardium. There was no difference in the total number of macrophages and neutrophils per high-power field between the starvation-derived extracellular vesicle (EV) group (*MVM*) and hypoxia-derived EV group (*HYP*) and the saline control group (*CON*).

After blocking with 5% nonfat dry milk in TBST (Tris-buffered saline with Tween; Boston BioProducts) for 1 hour, the membranes were incubated overnight at 4 °C with primary antibodies.[14] The membranes were washed, and horseradish peroxidase–conjugated secondary antibodies against mouse or rabbit (Cell Signaling) were prepared at a dilution of 2.5:10,000 in TBST containing 3% bovine serum albumin. Membranes were incubated with the secondary antibodies at room temperature for 1 hour,[14] then washed and processed for chemiluminescent detection and captured with a digital camera system (ChemiDoc MP; Bio-Rad).[14]

The membranes were imaged on a ChemiDoc Imaging System (Bio-Rad) using ECL Western Blotting Substrate (Thermo Fisher Scientific) as the developing agent. Membranes were stripped with Restore PLUS Western Blot Stripping Buffer (Thermo Fisher Scientific) to allow for repeat probing. All membranes were probed with glyceraldehyde 3-phosphate dehydrogenase or α-tubulin (Cell Signaling) to correct for loading error. Densitometric analysis of band intensity was performed using Image J software.[14]

Primary Antibodies

The primary antibodies were prepared by diluting them to a concentration of 1:1,000 in TBST solution containing 3% bovine serum albumin. The specific primary antibodies used in this study included those targeting interleukin (IL)-1 beta (Pro-IL-1β), IL-6, IL-10, IL-12, 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), all obtained from Cell Signaling.

Immunohistochemistry

The immunohistochemistry technique followed established protocol as described previously.[15] In summary, frozen section slides were thawed, fixed using 10% paraformaldehyde, blocked, and then incubated with a CD68 antibody (Cell Signaling). Images were captured at 20× magnification using a Nikon E800 Eclipse microscope. Macrophages were identified based on CD68 staining and quantified for each specimen in multiple high-power field (HPF), with an average count calculated per specimen. Neutrophils were identified based on neutrophil elastase staining, and their counts were quantified similarly. Representative images are provided in Figure 3.

Statistical Analysis

Statistical analyses was performed using Prism 9 (GraphPad Software). The normality of the data was assessed using the Shapiro-Wilk test. The Wilcoxon rank-sum test was used to analyze nonparametric data, and the Student *t* test was used to analyze normally distributed data. Results are presented as mean ± standard deviation (SD). For Western blot data, mean fold change was calculated and normalized to the average control value. Data points that deviated from the mean by >2 SD were excluded from the analysis, to ensure reliability of the statistical results. Immunohistochemistry for macrophages was determined by CD68 staining and reported as median macrophage count per HPF with interquartile range. Western blotting and immunohistochemistry data were statistically analyzed using the Wilcoxon rank-sum test. *P* values < .05 were considered significant.

RESULTS

Protein Expression

In the ischemic tissue obtained from the MVM group, a significant decrease in the expression of proinflammatory markers was observed compared to the CON group. Specifically, the MVM group exhibited significant reductions in

TABLE 1. Cytokine expression in ischemic myocardium of EV-treated swine

Protein	MVM group		HYP group	
	Median (IQR)	*P* value	Median (IQR)	*P* value
Proinflammatory				
NLRP3	0.74 (0.61-0.83)	.001	0.59 (0.59-0.72)	<.001
IL-6	0.84 (0.78-1.01)	.44	0.71 (0.61-0.78)	.03
IL-17	0.46 (0.35-0.60)	<.001	0.44 (0.43-0.73)	.003
Pro-IL-1ß	1.05 (0.99-1.15)	.77	0.47 (0.44-0.98)	.01
HLA-DRA	0.52 (0.40-0.59)	.002	0.60 (0.46-1.09)	.12
TNF-α	0.97 (0.57-2.39)	.99	1.39 (1.33-1.63)	.24
IFN-γ	0.83 (0.75-0.92)	.58	0.76 (0.66-1.13)	.88
IL-12	0.98 (0.93-1.02)	.88	1.10 (1.04-1.23)	.10
TLR2	0.53 (0.24-0.73)	.18	1.22 (0.60-1.37)	.99
NFκB	1.07 (1.01-1.17)	.44	0.85 (0.74-0.99)	.99
p-NFκB	1.11 (0.90-1.40)	.66	1.06 (0.87-1.14)	.98
Anti-inflammatory				
IL-10	0.49 (0.38-0.62)	.01	0.55 (0.42-0.67)	.24
IL-4	1.05 (0.82-1.71)	.99	0.95 (0.62-1.11)	.99
TGF-ß	0.79 (0.69-0.83)	.07	0.78 (0.55-1.04)	.16

Presented are immunoblotting results for all markers tested in the study. Immunoblotting data are reported as mean fold change in starvation-derived extracellular vesicle (EV) group (MVM; n = 10) and hypoxia-derived EV group (HYP; n = 7) with standard deviation (SD) normalized to average control (CON; n = 6). Data points >2 SD from the mean are excluded from the analysis. NOD-like receptor proteins (NLRP3), interleukin (IL)-6, IL-17, Pro-IL-1β, HLA class II histocompatibility antigen, DR alpha chain (HLA-DRA), tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), IL-12, Toll-like receptor 2 (TLR2), nuclear factor kappa light chain enhancer of activated B cells (NF-κB), IL-10, IL-4, and tumor growth factor beta (TGF-β). *MVM*, Starvation-derived extracellular vesicle group; *HYP*, hypoxia-derived EV group; *IQR*, interquartile range; *NLRP3*, NOD-like receptor proteins; *IL*, interleukin; *HLA-DRA*, HLA class II histocompatibility antigen, DR alpha chain; *TNF-α*, 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; *TGF-β*, tumor growth factor beta.

the expression levels of NLRP3 ($P < .01$), IL-17 ($P < .01$), and HLA-DRA ($P < .01$) (Table 1, Figure 4). The MVM group had reduced expression of the anti-inflammatory marker IL-10 ($P = .01$) compared to the CON group (Table 1).

HYP EV treatment resulted in a significant decrease in the expression of various proinflammatory markers compared to the CON group. Specifically, the HYP group demonstrated significantly reduced expression levels of IL-6 ($P = .03$), Pro-IL-1ß ($P = .01$), IL-17 ($P < .01$), and NLRP3 ($P < .01$) (Table 1, Figure 4).

No significant differences were observed in the expression levels of TNF-α, IFN-γ, IL-12, TLR2, and NFκB in either the MVM group or the HYP group (Table 1). Additionally, no significant correlations were found between coronary perfusion and cytokine concentration in either the MVM group or the HYP group, both at rest and with pacing, mitigating the influence of myocardial perfusion on the results (Table 2).

In the nonischemic tissue obtained from the MVM group, a significant decrease in the expression of proinflammatory markers was observed compared to the CON group (Figure 5). Specifically, the MVM group had significantly reduced expression levels of NLRP3 ($P < .05$), IL-17 ($P < .05$), HLA-DRA ($P < .05$), IL-6 ($P < .001$) and Pro-IL-1ß ($P = .05$) compared to the control group with no changes in the anti-inflammatory marker IL-10 (Figure 5), whereas HYP EVs showed only a significant decrease in NLRP3 ($P < .05$) compared to control in the nonischemic tissue (Figure 5). No significant differences between the HYP and CON groups were observed in the expression levels of HLA-DRA, Pro-IL-1ß, IL-17, IL-6, and IL-10 (Figure 5).

Macrophage and Neutrophil Counts

In ischemic myocardium, there were no statistically significant variations in the total number of macrophages and neutrophils per HPF between the 2 groups treated with EVs 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 (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 significantly reduced expression levels of 4 proinflammatory markers—IL-6, Pro-IL1ß, IL-17, and

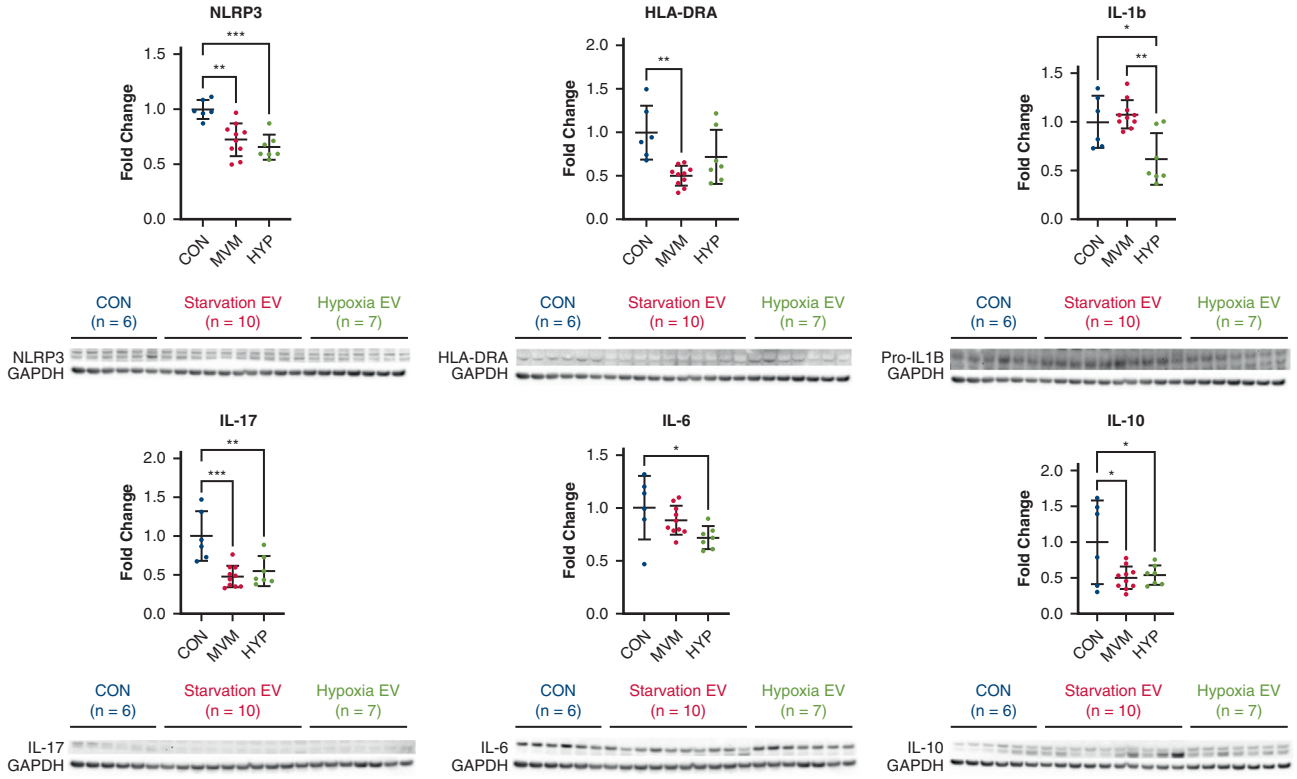

FIGURE 4. Relative protein expression in ischemic tissue by Western blot analysis normalized to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). Immunoblotting data is mean fold change in starvation-derived extracellular vesicle (*EV*) group (*MVM*; n = 10) and hypoxia-derived EV group (*HYP*; n = 7) with standard deviation (SD) normalized to average control (*CON*; n = 6). Data points >2 SD from the mean are excluded from the analysis. *NLRP3*, NOD-like receptor protein; *HLA-DRA*, HLA class II histocompatibility antigen; *IL*, interleukin. *$P < .5$; **$P < .01$; ***$P < .001$.

NLRP3—in the myocardial tissue treated with HYP EVs. On the other hand, MVM/standard EVs showed significantly decreased expression levels of 3 proinflammatory markers: 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 Pro-IL-1ß and IL-6, are known to have 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-1ß levels in the chronically ischemic myocardium of swine with metabolic syndrome after treatment with MVM EVs.[8] This reduction in proinflammatory signaling through EV therapy potentially could explain the observed improvements 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 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 present study confirms that both MVM EVs and HYP EVs effectively reduce Pro-IL-1ß levels, as shown in Figure 4, which displays protein expression of cytokines in the chronically ischemic myocardium (Table 1, Figure 4). Additionally, HYP EVs demonstrated a significant reduction in IL-6 levels compared to MVM EVs in the same myocardial tissue, whereas MVM EVs had no significant effect on IL-6 levels (Table 1, Figure 4). This reduced IL-6 expression 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 4). The stronger anti-inflammatory effect observed with HYP EVs may partially account for the previously reported increase in myocardial capillary density

TABLE 2. Correlation of coronary perfusion with cytokine concentration

Protein	At rest		With pacing (heart rate 150 bpm)	
	R value	*P* value	R value	*P* value
Proinflammatory				
NLRP3	<0.001	.92	0.02	.46
IL-6	0.002	.83	0.04	.34
IL-17	0.16	.05	0.07	.20
Pro-IL-1ß	0.19	.03	0.08	.18
HLA-DRA	0.001	.85	0.005	.73
TNF-α	0.04	.34	0.14	.07
IFN-γ	0.12	.10	0.007	.69
IL-12	0.02	.46	0.015	.57
TLR2	<0.001	.97	0.0015	.86
NFκB	0.005	.75	0.01	.63
p-NFκB	<0.001	.96	0.1	.12
Anti-inflammatory				
IL-10	0.13	.09	0.07	.19
IL-4	0.01	.63	0.17	.04
TGF-ß	0.009	.66	0.005	.74

There was no statistically significant correlation between coronary blood flow to the ischemic territory and the protein expression levels of NOD-like receptor proteins (NLRP3), interleukin (IL)-6, IL-17, Pro-IL-1β, HLA class II histocompatibility antigen, DR alpha chain (HLA-DRA), tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), IL-12, Toll-like receptor 2 (TLR2), nuclear factor kappa light chain enhancer of activated B cells (NF-κB), IL-10, IL-4, and tumor growth factor beta (TGF-β) in extracellular vesicle-treated pigs, both at rest and during pacing. *NLRP3*, NOD-like receptor proteins; *IL*, interleukin; *HLA-DRA*, HLA class II histocompatibility antigen, DR alpha chain; *TNF-α*, 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; *TGF-β*, tumor growth factor beta.

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 significantly reduced inflammation in the myocardial tissue of both the starvation-derived and hypoxia-derived EV groups may provide insight 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] Although 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 needed 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 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 injuries, holds promise as a therapeutic strategy.[24] Further research in this area could provide new insight 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 proinflammatory and anti-inflammatory markers in the context of chronic myocardial ischemia. The reduction in proinflammatory markers in the MVM group and the significant decreases in multiple proinflammatory 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 reducing proinflammatory

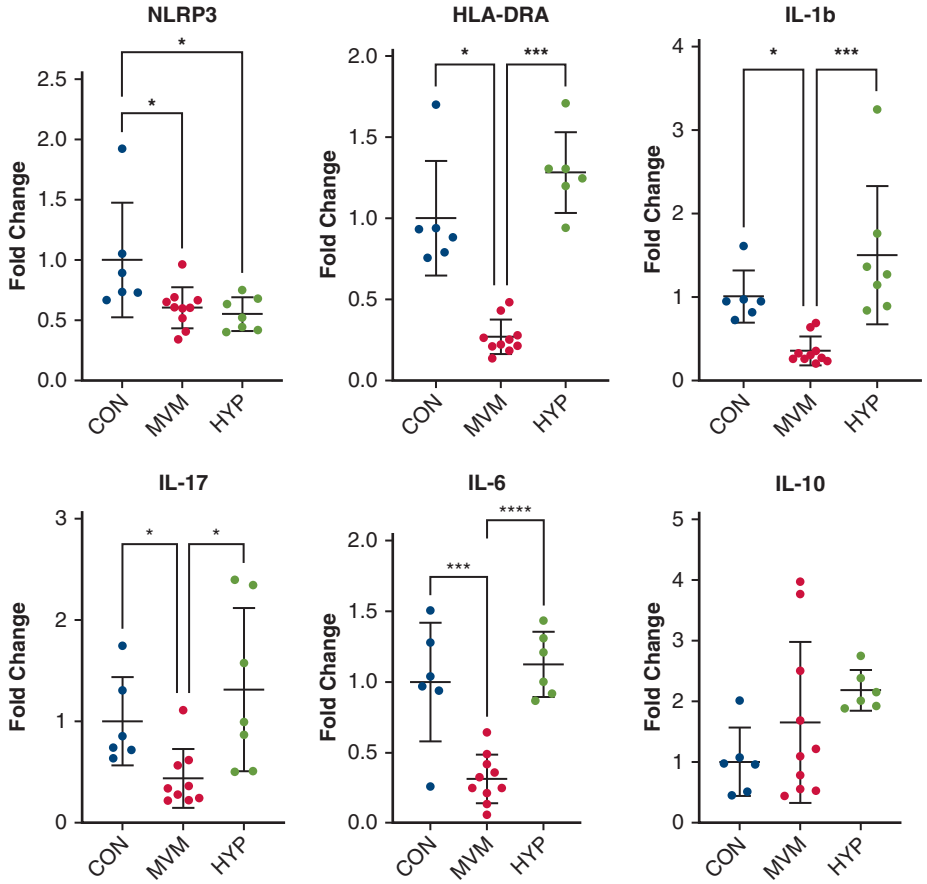

FIGURE 5. Relative protein expression in nonischemic tissue by Western blot normalized to glyceraldehyde 3-phosphate dehydrogenase. Immunoblotting data are expressed as mean fold change the in starvation-derived extracellular vesicle (EV) group (*MVM*; n = 10) and hypoxia-derived EV group (*HYP*; n = 7) with standard deviation (SD) normalized to the average control group (*CON*; n = 6). Data points >2 SD from the mean are excluded from the analysis. *NLRP3*, NOD-like receptor protein; *HLA-DRA*, HLA class II histocompatibility antigen; *IL*, interleukin. **P* < .5; ***P* < .01; ****P* < .001.

markers in the ischemic tissue compared with nonischemic tissue, whereas the MVM EVs were more effective in the nonischemic tissue compared to ischemic tissue (Figures 4 and 5). Further investigations are needed to elucidate the underlying mechanisms and the clinical implications of these findings, as further discussed in a study by Sabe and colleagues.[13]

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. Further studies by our lab into the transcriptomic profile of the EVs will provide better mechanistic clarity.

This study is subject to some limitations, including 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]

CONCLUSIONS

Our study demonstrates that both starvation- and hypoxia-preconditioned EVs exert a significant reduction in inflammation in the myocardium of swine with chronic ischemia (Table 1, Figure 4). This anti-inflammatory effect is independent of coronary perfusion, indicating that the observed decrease in inflammation is not attributed solely to improved blood flow (Table 2). The findings suggest that EVs derived from hypoxic conditions (HYP EVs) may have a superior anti-inflammatory effect compared to those derived from normoxic conditions (MVM EVs) in the context of chronic myocardial ischemia.

Conflict of Interest Statement

The authors reported no conflicts of interest.

The *Journal* policy requires editors and reviewers to disclose conflicts of interest and to decline handling or reviewing manuscripts for which they may have a conflict of interest. The editors and reviewers of this article have no conflicts of interest.

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