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ORIGINAL ARTICLE

Poloxamer 188 Protects Isolated Adult Mouse Cardiomyocytes from Reoxygenation Injury

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Abstract

Reperfusion injury is a complex pathological event involving processes that can lead to further disruption of the cell membrane and function following an ischemic event. Return of blood flow allows for the needed reperfusion; however, for a period of time before remaining viable cells stabilize, reperfusion results in additional cellular injury. In cardiomyocytes, loss of membrane integrity allows abnormal influx of extracellular calcium, leading to hyper-contracture and cell death. Methods to improve the membrane integrity of cardiomyocytes overwhelmed by pathological disruptions, such as reperfusion injury, are needed to prevent cell death, because of the myocardium's limited ability to regenerate. Research has shown administration of the copolymer P(oloxamer) 188 before ischemia/reperfusion can protect cardiomyocytes through membrane stabilization. This study sought to determine whether the administration of P188 at the beginning of the clinically more relevant time of reperfusion after ischemia will attenuate any additional damage to cardiomyocytes by stabilizing membrane integrity to allow the cells to maintain function. Using an in-vitro cardiomyocyte model subjected to hypoxia/reoxygenation to simulate ischemia/reperfusion injury, we show that reoxygenation significantly potentiates the injury caused by hypoxia itself. P188, with its unique combination of hydrophobic and hydrophilic chemical properties, and only delivered at the beginning of reoxygenation, dose-dependently protected cardiomyocytes from injury due to reoxygenation by repairing cell membranes, decreasing calcium influx, and maintaining cellular morphology. Our study also shows the hydrophobic portion of P188 is necessary for the stabilization of cell membrane integrity in providing protection to cardiomyocytes against reoxygenation injury.

KEYWORDS

cell membrane stabilizer, heart, ischemia, polyethylene glycol, reperfusion injury, tri-block copolymer

Abbreviations: AU, absorbance unit; C/N, control/normoxia; Ca^{2+}, calcium; CCMS, copolymer-based cell membrane stabilizer; CPR, cardiopulmonary resuscitation; Em, emission wavelength; Ex, excitation wavelength; H, hypoxia only; H/R, hypoxia/reoxygenation; I/R, ischemia/reperfusion; LDH, lactate dehydrogenase; MI, myocardial infarction; MW, molecular weight; P188, Poloxamer 188; PEG, polyethylene glycol; PEO, polyethylene oxide; PPO, polypropylene oxide; RFU, relative fluorescence unit; SGF, serum- and glucose-free; STEMI, ST-elevation myocardial infarction.

1 | INTRODUCTION

Reperfusion injury is an unavoidable consequence of planned cardiac procedures, including coronary artery bypass graft surgeries, and unplanned cardiac events, including primary coronary interventions for acute coronary syndromes and cardiac arrests with cardiopulmonary resuscitation (CPR). It follows an ischemic period, a disruption of blood flow that limits the availability of oxygen and nutrients, causing cellular consequences such as hypoxia, decreased intracellular pH, altered ion exchange/transport (eg, excessive influx of calcium [Ca^{2+}] ions), cell swelling, membrane disruptions, and cell death.[1-4] Reperfusion is the needed return of blood flow, oxygen, and nutrients. However, reperfusion itself, for a brief period before the remaining viable cells can stabilize, results in additional cellular dysfunctions, including production of reactive oxygen species, further membrane damage, increased intracellular Ca^{2+} ($[Ca^{2+}]_i$), altered redox state, hyper-contracture, mitochondrial dysfunction, and cell death, all of which have been observed in several cardiac-related cell types, including cardiomyocytes.[1-5]

A critical focus of clinical interventions is to improve survival rates and quality of life for thousands of patients each year by attenuating the reperfusion injury that can occur when organs and tissues are knowingly reperfused after an ischemic event. Unfortunately, there is no current therapy available for combating reperfusion injury. A variety of interventions have been investigated to limit reperfusion injury following ischemia, but the majority of this research aims to develop therapies to be provided before the ischemia occurs. Few studies are focusing strictly on strategies to be given at the clinically more relevant reperfusion phase after ischemia, to target any salvageable cells. Ischemic post-conditioning, for example, the intentional use of "stuttered" reperfusion, during the first minutes of reperfusion, has been successfully employed in models of myocardial infarction (MI) and cardiac arrest.[6-8] Reperfusion injury can also be attenuated by pharmacological post-conditioning; volatile anesthetics, such as sevoflurane, when given immediately upon reperfusion for several minutes, have been shown to improve functional recovery in hearts.[9,10] Unfortunately, results of studies using these interventions have been mixed in the clinical setting.[11,12]

Another therapeutic option may involve methods to improve the membrane integrity and resealing ability of cells that are overwhelmed due to pathological disruptions, especially since the myocardium has a limited ability to regenerate.[13] Copolymer-based cell membrane stabilizers (CCMS) have this potential. CCMS, or Poloxamers, are synthetic non-ionic block copolymer molecules available in varying molecular weights (MW) and ratios of hydrophobic polypropylene oxide (PPO) to hydrophilic polyethylene oxide (PEO) chains. Poloxamer 188 (P188) is a flexible tri-block CCMS composed of single bonds and a central hydrophobic 30 PPO unit chain that covalently connects two hydrophilic 75 PEO unit chains (Figure 1A), making P188 ~20% hydrophobic with an average MW of 8400 g/mol.[14,15] Through work using X-ray scattering, atomic force microscopy, and computer simulations, it is thought that P188 directly seals and stabilizes cell membranes by inserting the hydrophobic portion into damaged areas of the membrane while the hydrophilic chains interact with the lipid headgroups of the bilayer and remain at the surface of the membrane, ultimately closing the tear.[16-18]

Thus, our study was based on the hypothesis that P188, with its unique hydrophobic/hydrophilic chemical properties, and given only on reoxygenation, can protect cardiomyocytes against further injury from reoxygenation following hypoxia by stabilizing the damaged cell membrane, and allowing remaining viable cells to maintain function. Our study's objectives were to (a) perform dose response experiments to determine the protective concentration of P188 when administered to isolated cardiomyocytes at the start of reoxygenation following hypoxia (simulated ischemia/reperfusion [I/R] injury) and (b) determine potential protective mechanisms of P188 when provided to isolated cardiomyocytes only during reoxygenation.

The study results in our in-vitro adult mouse cardiomyocyte model show that reoxygenation potentiates the injury caused by hypoxia itself, that P188 given only at the start of reoxygenation dose-dependently protects the isolated cells against further injury following hypoxia, and that the hydrophobic portion of P188 is necessary for the protection provided to the cells. This information may assist in better defining P188's mechanism of action and the importance of its hydrophobic component when the molecule is administered as a protective compound against I/R injuries.

2 | MATERIALS AND METHODS

An ethical statement is not required as no human or animal research was conducted.

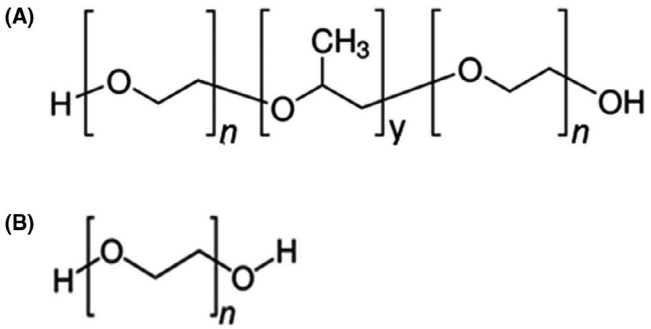

FIGURE 1 A, Diagram showing the general structure of a tri-block copolymer. P188 (MW 8400 g/mol) has a central portion containing 30 PPO units (represented by y in the diagram) flanked by two side chains consisting of 75 PEO units each (represented by n in the diagram). B, Diagram showing the general structure of a PEG molecule. PEG is a polymer consisting of varying numbers of PEO units (number of units represented by n in the diagram) that make up varying MWs. PEG (MW 8000 g/mol) was used as the control molecule to P188 in our study

2.1 | Cell culture

Isolated cardiomyocytes from ventricles of adult male C57BL/6J mouse hearts, as well as the culture media, passaging solutions, and flasks and plates precoated with a proprietary laminin-based extracellular matrix for cell adherence, were obtained from Celprogen (Torrance, CA, USA). The cardiomyocytes were maintained under Celprogen-specified culture conditions, such as regular cardiomyocyte growth media containing 10% fetal bovine serum (FBS), 5 mmol/L glucose, and antibiotics, and incubation of the cells in a standard culture environment of humidified 21% O_2: 5% CO_2: 74% N_2 at 37°C. Under these conditions, the cardiomyocytes retain the majority of biochemical and molecular properties of in-vivo cardiomyocytes, such as expression of α-adrenoreceptors and troponin, and morphologically show the expected rod shape (Figure 2A) until the cells begin to spread to create cell-cell contacts and generate a confluent layer with cells becoming more rounded in shape (Figure 2B). Confluent cultures of cardiomyocytes were used to better mimic the normal in-vivo state of these cells in the heart, and cells from adult mice were chosen to better represent cardiomyocytes in adult human hearts.

For experiments, cardiomyocytes between passages 5 and 10, and from three different lots of cells, were plated in regular media at a density of 50 000 cells/cm^2 into 96-well black-walled, clear-bottom plates, then placed in a standard culture environment. Four to six replicate wells for each control and treatment group were plated in each experiment. After ~72 hours, when the cardiomyocytes reached confluency, the plates of cells for each experiment were randomized to control/normoxia (C/N) or hypoxia/reoxygenation (H/R) conditions.

2.2 | In-vitro *H/R injury*

To simulate I/R, the cardiomyocytes were exposed to serum- and glucose-free (SGF) media in a humidified Billups-Rosenthal plexiglass hypoxia chamber (Stemcell Technologies; Vancouver, BC, Canada) filled with the hypoxic gas mixture (0.01% O_2: 5% CO_2: 94.99% N_2) and placed in a 37°C incubator for 5 hours. The ability to maintain the hypoxic 0.01% O_2 level was previously tested with a microprocessor-based O_2 sensor. The 2 hours of reoxygenation began when the cells were removed from the hypoxic chamber, the media replaced with regular serum- and glucose-containing media, and the experimental plates returned to a standard culture environment (21% O_2). To control for any effects of media replacement, the media of the corresponding plates of C/N cells was changed at the same times the media of the H/R cells was changed; from normal media to SGF media for the hypoxic period and back to normal media for the reoxygenation phase.

2.3 | Optimization of H/R conditions for confluent cultures of mouse cardiomyocytes

Previous studies with adult human cardiomyocytes at 80% confluency showed 40%-60% damage in cell number and viability, as well as lactate dehydrogenase (LDH) release, after 3 hours of hypoxia (0.1% O_2) in SGF media followed by 2 hours of reoxygenation.[19] To optimize the H/R protocol for the confluent cultures of adult mouse cardiomyocytes, we performed a series of experiments to replicate the damage observed in the non-confluent cultures of human cardiomyocytes. This amount of damage would allow examination of the protective actions of a given agent (eg, P188)

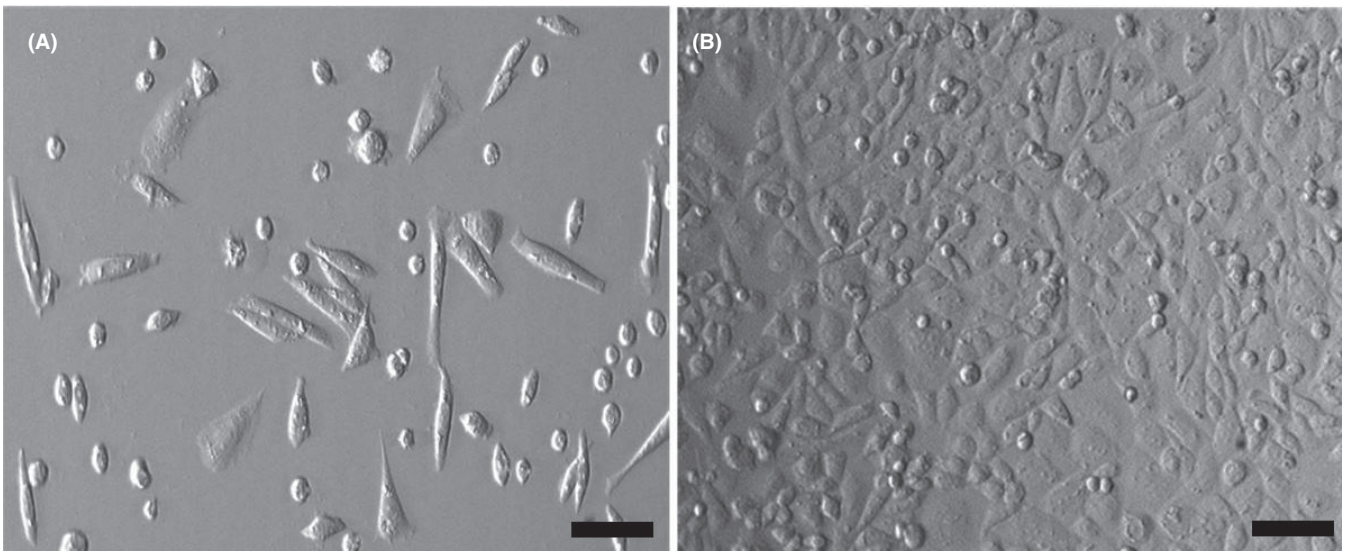

FIGURE 2 A, Representative photo of the general appearance of the isolated adult mouse cardiomyocytes 6 h after plating. At least 50% of the cells show the expected rod-shaped morphology of cardiomyocytes. B, Representative photo of the typical appearance of the cardiomyocytes ~72 h after plating. A confluent layer has formed, with the cells becoming more rounded due to increased cell-cell contact and limited growth area. Scale bars are 100 μm in length

over the linear segment of the cytotoxicity response curve. It was determined that the optimal parameters to replicate the same cytotoxicity response observed in the non-confluent human cardiomyocytes was 5 hours of hypoxia at 0.01% O_2 in SGF media followed by 2 hours of reoxygenation with regular media for the confluent mouse cardiomyocytes. We speculate that the requirement of a significantly greater and longer hypoxia challenge is due to two factors: (1) confluent cardiomyocyte cultures rely on glycolysis rather than aerobic respiration[20] and (2) these isolated mouse cardiomyocytes are not consuming adenosine triphosphate by contracting and relaxing.

2.4 | P188 dose-response curves

The therapeutic potential of P188 administered at the start of reoxygenation was tested at five logarithmic concentrations ranging from 10 μmol/L to 1 mmol/L. This range was chosen based on other studies that have investigated the cellular protective potential of P188; in mouse muscle myoblast cultures (~80% confluent) exposed to hypo-osmotic stress/isotonic recovery, 14 μM P188 was protective;[21] 150 μmol/L P188 fully restored dystrophic mouse myocyte stretch compliance;[22] and 0.5 mmol/L P188 "repaired" cultured astrocytes that had been exposed to traumatic brain injury-induced microcavitation.[23]

Polyethylene glycol (PEG) with a MW of 8000 g/mol was used as a control molecule. This PEG is considered the optimal control for P188 since it has a MW close to that of P188, produces similar osmolarity, but is purely hydrophilic (Figure 1B).

P188 and PEG were purchased from Millipore Sigma (St. Louis, MO, USA). They were dissolved in regular cardiomyocyte media to achieve the desired concentrations (10 μmol/L - 1 mmol/L). At these concentrations, both are ≥ 96% soluble, do not affect the pH of the media, and exist as a dynamic solution of single molecules, as well as grouped micelles.[17]

2.5 | Assessment of cell number and viability

As done in other studies, cell number and viability were assessed in all experiments using the CyQUANT Direct Cell Proliferation Assay Kit (Molecular Probes, Inc, Eugene, OR, USA).[24-26] The kit fluorescently measures the DNA content only in healthy cells while blocking the staining of dead cells and cells with compromised membranes. Since DNA content is highly regulated, cell number estimates, as well as cytotoxicity assessments, are very accurate.[24]

At the end of each experiment (ie, the 2-hour reoxygenation period; except for H only experimental groups), the cardiomyocytes were washed with 1x phosphate-buffered saline (PBS) before 100 μL of fresh regular media was added back to the wells. An equal volume of prepared 2x CyQUANT detection buffer was added, and the cells incubated for 60 minutes at 37°C. The fluorescence of each well was read from the bottom at an excitation (Ex) of 480 nm and emission (Em) of 535 nm using a plate reader (Synergy H1; BioTek Instruments Inc, Winooski, VT, USA).

In a subset of experiments using P188, cell number and viability were also assessed using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTS; Promega, Madison, WI, USA). The tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] in this assay is reduced into a colored formazan product by dehydrogenase enzymes in metabolically active cells. At the end of each experiment, 20 μL of assay reagent was added to each well and the plates incubated at 37°C for 2 hours. Absorbance of each well was measured from the top at 490 nm using the plate reader.

The viability of the cardiomyocytes in relation to apoptotic cell death was assessed in another subset of P188 experiments using the Caspase-3 Assay Kit (BioVision Research Products, Mountain View, CA, USA). In viable cells, caspase-3 activity is very low, but increases with processes associated with apoptosis. At the end of each experiment, following the assay protocol, the cells were lysed, the cell lysates combined with the kit reagent and DEVD substrate, and incubated at 37°C for 1 hour. Absorbance of each well was measured from the top at 405 nm using the plate reader.

2.6 | Assessment of cell membrane injury and repair

Cell membrane injury and repair were assessed two ways in all experiments. The first method was the measurement of the intracellular enzyme LDH released from damaged cells into the culture media using the LDH Cytotoxicity Assay Kit (Pierce Biotechnology, Rockford, IL, USA). The leakage of LDH is widely used as an indicator of reduced cell membrane integrity and cytotoxicity.

At the end of each experiment, 50 μL of media from each well to be used to assess cell number/viability by the CyQUANT assay was transferred to a corresponding well of a new 96-well plate and mixed with 50 μL of prepared LDH reaction mixture. After a 30-minute incubation at room temperature, the reactions were terminated by the addition of 50 μL of stop solution, which halts the reduction of the tetrazolium salt to the formazan product. Absorbance of each well was measured from the top at 490 nm using the plate reader.

The membrane impermeant styryl dye FM1-43 (Molecular Probes, Inc) was utilized as an additional measure of cell membrane damage, as well as repair, since it has been used for these assessments in a range of other cell systems.[22,27] FM1-43 remains extracellular unless damage to the membrane allows it to become incorporated into the lipid bilayer of the cell membrane, where it specifically fluoresces.[28]

Following the method of Yasuda et al,[22] 2.5 μmol/L FM1-43 was added to the media at the start of the reoxygenation period. At the end of the experiment, the cells were washed with 1x PBS

to remove any remaining extracellular, non-incorporated dye, then 100 μL of 1x PBS was added to the wells and the fluorescence at Ex = 488 nm and Em = 568 nm was read from the bottom using the plate reader.

2.7 | Measurement of intracellular [Ca^{2+}]

$[Ca^{2+}]_i$ was assessed using the Fluo-4 Direct Calcium Assay Kit (Molecular Probes, Inc). Fluo-4 is a fluorescent in-cell Ca^{2+} indicator. The kit also utilizes a suppression dye to reduce background fluorescence, and probenecid to inhibit transport of the internalized Fluo-4 outside the cells.

Prior to the start of each experiment, cells were loaded with Fluo-4 by adding 100 μL of the prepared 2x Fluo-4 Direct loading solution to the wells containing cells and 100 μL of regular culture media. Following a 60-minute incubation at 37°C, the cells were washed with 1x PBS to remove any remaining non-internalized Fluo-4. The appropriate experimental media (100 μL/well), regular culture media for C/N plates and SGF media for H/R plates, was then added and the experiment conducted. At the end of each experiment, the fluorescence of each well was read from the bottom at Ex = 494 nm and Em = 576 nm using the plate reader.

2.8 | Cellular morphology assessment

Cellular morphology images were captured for the experimental groups using differential interference contrast microscopy (Leica DM IL LED) and Leica Application Suite X (LAS X), version 2.0, software (Leica Microsystems, Inc, Buffalo Grove, IL).

2.9 | Statistics

All experiments were repeated at least three times (N = 3; where each N represents the average of each set of treatment wells[4-6] per experiment). Results are shown as dot plots, with individual experiment data points represented together with the average of all replicates ± the standard error of the mean (SEM) bars. Statistical analyses were performed using Prism 6 software (GraphPad Software, San Diego, CA, USA). Normal distribution of data was assessed by the Kolmogorov-Smirnov test. Statistical differences between normally distributed data groups were analyzed by one-way analysis of variance followed by Student-Newman-Keuls post-hoc comparisons. Data groups found to be not normally distributed were analyzed by the nonparametric Kruskal-Wallis with Dunn's post-hoc Dunn's test. Logarithmic regression analyses were performed to determine dose-dependency of P188 concentration effects using Excel (Microsoft Office 2019). A P value of <.05 was considered to indicate statistically significant differences (two-tailed).

3 | RESULTS

As per data sharing guidelines, original data are kept with the corresponding author and will be made available on a case-by-case basis upon request.

3.1 | Reoxygenation potentiates cardiomyocyte injury caused during hypoxia

Two hours of reoxygenation following 5 hours of hypoxia resulted in a significant increase in cytotoxicity compared to hypoxia alone. Hypoxia significantly reduced cell number/viability by ~40%, and reoxygenation further reduced cell number/viability an additional 23%, or by nearly 63%, compared to C/N (Figure 3A). Corresponding results were obtained in studies assessing cell membrane damage/repair, influx of Ca^{2+}, and cellular morphology. Hypoxia significantly increased LDH release by 65% over C/N levels, while reoxygenation further increased LDH release to 76% compared to C/N (Figure 3B). Cell membrane damage, assessed by FM1-43 dye incorporation, was significantly increased by ~42% due to hypoxia, and an increase of ~58% was observed following reoxygenation, compared to C/N (Figure 3C). Measurement of $[Ca^{2+}]_i$ revealed a significant increase by ~54% with hypoxia, and an ~67% increase with reoxygenation, compared to C/N (Figure 3D). Finally, representative photos of each group (C/N, H only, and H/R) show what was consistently observed in the experiments conducted (Figure 3E). In the C/N group, the cells are confluent and show normal cellular morphology at the end of an experiment. The H only group shows irregularly shrunk and rounded cells, as well as larger areas where no cells are present. Reoxygenation caused even larger areas without cells, and more cells became irregularly shrunk, rounded, and even disintegrated. These data confirm that reoxygenation following hypoxia in our model produces further significant increases in cellular damage relative to hypoxia alone. This reoxygenation injury is the focus of our studies with P188.

3.2 | P188 protects cell number/viability from reoxygenation injury

Hypoxia itself significantly decreases cell number/viability (as assessed by the CyQUANT assay) compared to C/N conditions, and reoxygenation causes a further significant decrease in our model. With P188 present during reoxygenation, a dose-dependent increase was seen with concentrations from 10 μmol/L to 100 μmol/L, while concentrations from 30 μmol/L to 1 mmol/L significantly increased cell number/viability in H/R conditions. In fact, these concentrations improved cell number/viability to above the average hypoxia only level of ~72 000 cells/well (indicated by the dashed line in Figure 4A and B), thus abolishing reoxygenation injury (Figure 4A). No significant effect of any P188 concentration was observed under C/N conditions (Figure 4A). In experiments with PEG, no protective effects

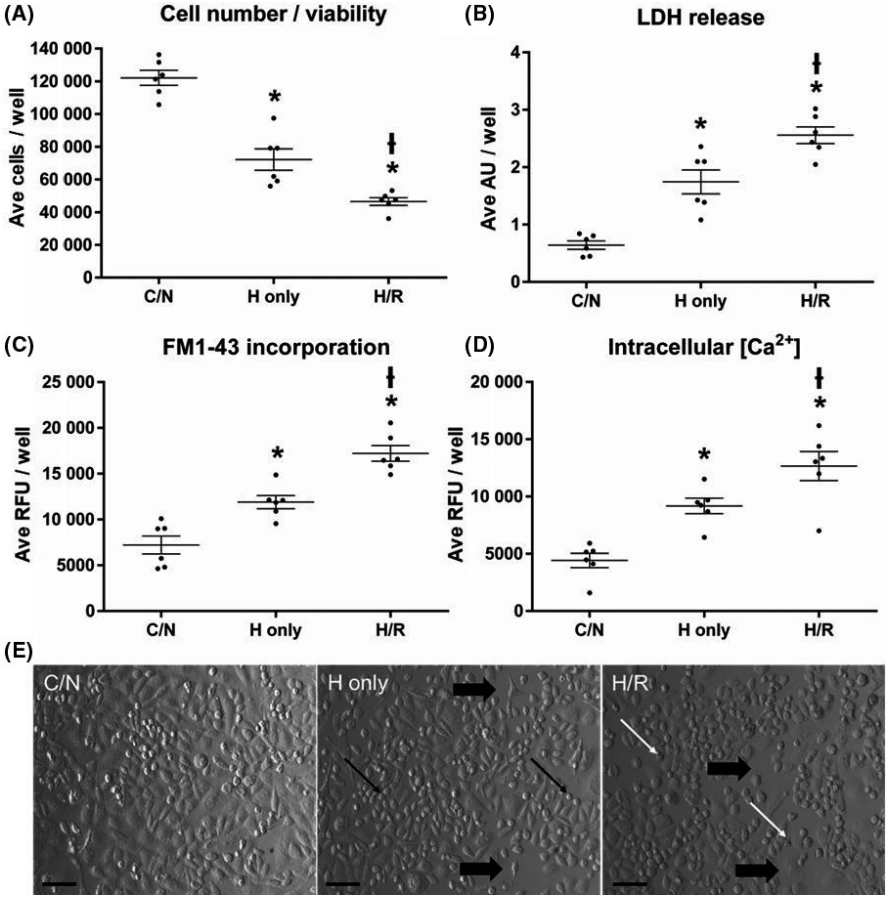

FIGURE 3 Potentiation of injury by reoxygenation following hypoxia in the five main indices of cellular function/dysfunction assessed. Reoxygenation following hypoxia significantly decreases (A) cell number/viability as assessed by the CyQUANT Direct Cell Proliferation Assay Kit (data expressed as dot plots of individual experiment data points together with the average number of cells per well ± the standard error of the mean [SEM] bars, number of experiments [N] = 6, 4-6 replicate wells per treatment per experiment, *vs C/N, ł vs H only), and significantly increases (B) LDH release (data expressed as dot plots of individual data points together with the average absorbance units [AU] per well ± SEM bars, N = 6, 4-6 replicate wells per treatment per experiment, *vs C/N, ł vs H only), (C) membrane damage as assessed by FM1-43 incorporation (data expressed as dot plots of individual data points together with the average relative fluorescent units [RFU] per well ± SEM bars, N = 6, 4-6 replicate wells per treatment per experiment, *vs C/N, ł vs H only), and (D) $[Ca^{2+}]_i$ (data expressed as dot plots of individual data points together with the average RFU ± SEM bars, N = 6, 4-6 replicate wells per treatment per experiment, *vs C/N, ł vs H only. (E) Representative photos of each group (C/N, H only, H/R) show what was consistently observed in the experiments conducted; C/N group shows the normal condition and morphology of the cells at the end of the experiment, the H only group shows irregularly shrunk and rounded cells (small black arrows) and larger areas with no cells present (large black arrows), the H/R group shows more areas with no cells present, irregularly shrunk and rounded cells, as well as disintegrated cells (small white arrows). Scale bars are 100 μm in length

were observed on cell number/viability under either C/N or H/R conditions (Figure 4B).

In a subset of experiments with P188 (N = 5), cell number/viability was also assessed by the activity of dehydrogenase enzymes (using the MTS assay) to confirm the results using the CyQUANT assay. H/R significantly decreased cell number/viability compared to C/N conditions. The P188 concentrations of 100 μmol/L - 300 μmol/L present during reoxygenation significantly increased cell number/viability in H/R conditions (Figure 4C). No significant effect of any P188 concentration was observed under C/N conditions (Figure 4C).

In another small subset of experiments with P188 (N = 3), cell viability was assessed by the activity of the apoptosis-initiating enzyme caspase-3. These three experiments showed a trend toward increased caspase-3 activity in cells exposed to H/R compared to C/N conditions. With P188 present during reoxygenation, concentrations of 100 μmol/L - 1 mmol/L showed a reduction in the amount of caspase-3 activity, with 100 μmol/L and 300 μmol/L causing the greatest reduction in activity (Figure 4D). No effect of any P188 concentration was observed under C/N conditions (Figure 4D).

3.3 | P188 reduces cell membrane damage during reoxygenation

In the first measure of cell membrane damage/repair, there was a significant, approximately 4-fold increase in LDH release from cells exposed to H/R compared to C/N. While all concentrations of P188 present during reoxygenation decreased LDH release to some

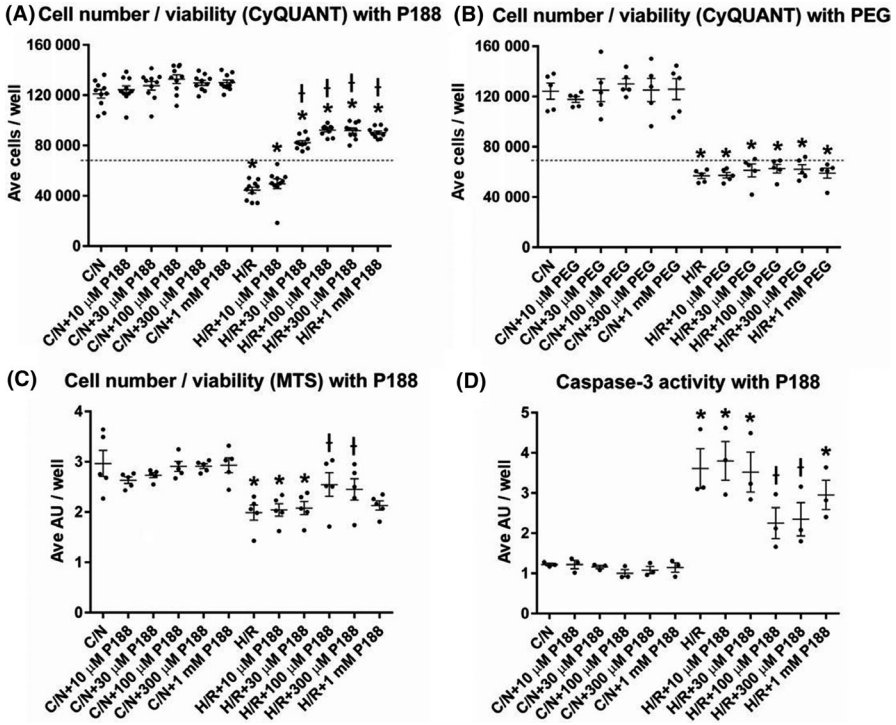

FIGURE 4 Assessment of cell number and viability. (A) Using the CyQUANT assay, the significant decrease in cell number/viability during H/R compared to cells under C/N conditions was significantly attenuated with P188 concentrations of 30 μmol/L - 1 mmol/L. The dashed line represents the average hypoxia only level of ~72 000 cells/well. No significant effect of P188 was observed under C/N conditions. Data expressed as dot plots of individual data points together with the average number of cells/well ± SEM bars, N = 10, 4-6 replicate wells per treatment per experiment, *vs C/N, ł vs H/R media only. (B) No significant effect of PEG on cell number/viability as assessed by the CyQUANT assay was observed under C/N or H/R conditions. Data expressed as dot plots of individual data points together with the average number of cells/well ± SEM bars; N = 5, 4-6 replicate wells per treatment per experiment, *vs C/N. (C) Using the MTS assay as another assessment of cell number/viability in a subset of experiments with P188, the significant decrease in cell number/viability during H/R compared to cells under C/N conditions was significantly attenuated with 100 μmol/L and 300 μmol/L P188. No significant effect of P188 was observed under C/N conditions. Data expressed as dot plots of individual data points together with the average AU/well ± SEM bars, N = 5, 4-6 replicate wells per treatment per experiment, *vs C/N, ł vs H/R media only. (D) Measurement of caspase-3 activity was used as an additional assessment of cell viability in another subset of experiments with P188. An increase in caspase-3 activity during H/R was observed compared to cells in C/N conditions. P188 concentrations of 100 μmol/L and 300 μmol/L showed a trend in decreasing caspase-3 activity. No effect of P188 was observed under C/N conditions. Data expressed as dot plots of individual data points together with the average AU/well ± SEM bars, N = 3, 4-6 replicate wells per treatment per experiment, *vs C/N, ł vs H/R media only

extent in H/R conditions, a dose-dependent decrease was observed with P188 concentrations from 10 μmol/L to 100 μmol/L, and in the majority of experiments 100 μmol/L to 1 mmol/L P188 significantly decreased LDH levels to below that released during hypoxia only (~1.7 average absorbance units [AU]/well; indicated by dashed lines in Figure 5A and B), and so abolished the reoxygenation injury (Figure 5A). No significant effect of any P188 concentration was observed under C/N conditions (Figure 5A). With PEG, no protective effects on LDH release were observed under either C/N or H/R conditions (Figure 5B).

With the assessment of cell membrane damage/repair by FM1-43 incorporation, there was a significant ~2.5-fold increase in cell membrane damage during H/R compared to C/N. P188 concentrations from 10 μmol/L to 300 μmol/L dose-dependently decreased FM1-43 incorporation when present during reoxygenation following hypoxia, and concentrations of 30 μmol/L - 1 mmol/L significantly decreased FM1-43 incorporation. In most experiments, P188 concentrations of 100 μmol/L to 1 mmol/L significantly decreased FM1-43 incorporation to amounts equal to or below that caused by hypoxia only (~11 900 average relative fluorescence units (RFU]/well; indicated by the dashed line in Figure 5C and D), thus attenuating the reoxygenation injury (Figure 5C). No significant effect of any P188 concentration was observed under C/N conditions (Figure 5C). Under both C/N and H/R conditions, no protective effects by PEG on FM1-43 incorporation were observed (Figure 5D).

3.4 | P188 reduces $[Ca^{2+}]_i$ during reoxygenation

There was a significant ~3-fold increase in $[Ca^{2+}]_i$ in cells during H/R compared to C/N. When present during reoxygenation, P188 concentrations from 10 μmol/L to 300 μmol/L dose-dependently decreased the influx of Ca^{2+}, while 100 μmol/L and 300 μmol/L significantly decreased $[Ca^{2+}]_i$ under H/R conditions. Additionally, in

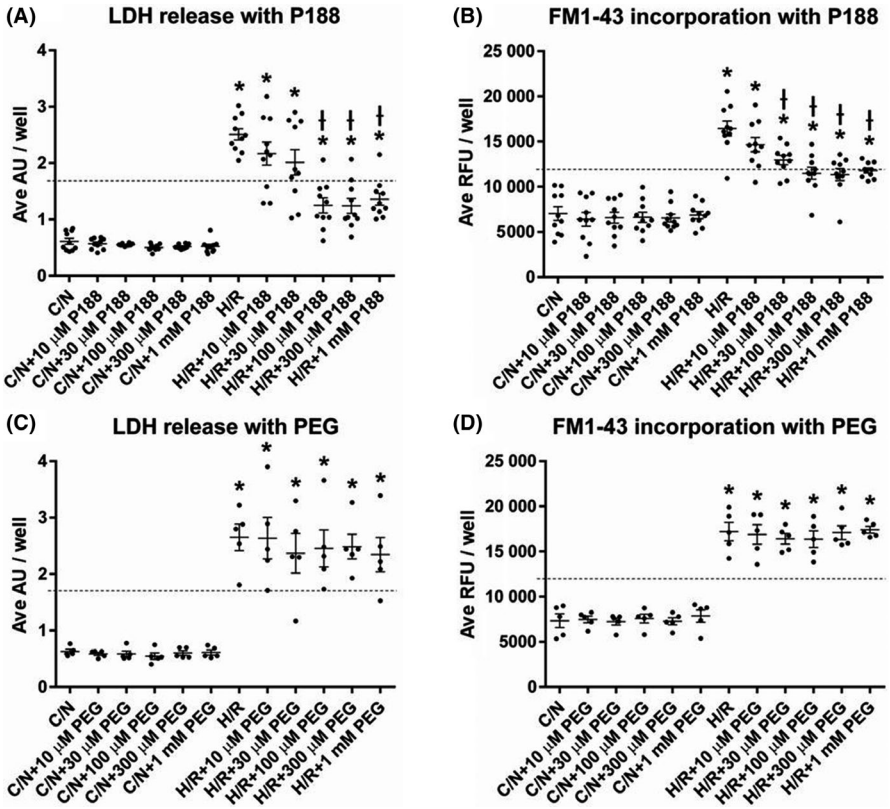

FIGURE 5 Assessment of cell membrane injury and repair. (A) The significant increase in LDH release during H/R compared to cells under C/N conditions was significantly decreased with P188 concentrations of 100 μmol/L - 1 mmol/L. The dashed line represents the average hypoxia only level of ~1.7 AU/well. No significant effect of P188 was observed under C/N conditions. Data expressed as dot plots of individual data points together with the average AU/well ± SEM bars, N = 10, 4-6 replicate wells per treatment per experiment, *vs C/N, ł vs H/R media only. (B) No significant effect of PEG on LDH release was observed under C/N or H/R conditions. Data expressed as dot plots of individual data points together with the average AU/well ± SEM bars, N = 5, 4-6 replicate wells per treatment per experiment, *vs C/N. (C) The significant increase in FM1-43 incorporation during H/R compared to cells under C/N conditions was significantly decreased with P188 concentrations of 30 μmol/L - 1 mmol/L. The dashed line represents the average hypoxia only level of ~11 900 RFU/well. No significant effect of P188 was observed under C/N conditions. Data expressed as dot plots of individual data points together with the average RFU/well ± SEM bars, N = 10, 4-6 replicate wells per treatment per experiment, *vs C/N, ł vs H/R media only. (D) No significant effect of PEG on FM1-43 incorporation was observed under C/N or H/R conditions. Data expressed as dot plots of individual data points together with the average RFU/well ± SEM bars, N = 5, 4-6 replicate wells per treatment per experiment, *vs C/N

most experiments conducted, these two P188 concentrations also brought $[Ca^{2+}]_i$ levels below that caused by hypoxia only (~9200 average RFU/well, indicated by the dashed line in Figure 6A and B), thus abolishing reoxygenation injury (Figure 6A). No significant effect of any P188 concentration was observed under C/N conditions (Figure 6A). Under both C/N and H/R conditions, no protective effects by PEG on $[Ca^{2+}]_i$ were observed (Figure 6B).

3.5 | P188 protects cellular morphology from reoxygenation injury

Representative photos (Figure 7) show that there was a noticeable decrease in the number of cells with normal cellular morphology in the H/R groups compared to cells in the C/N groups that was consistently observed in all conducted experiments. Many H/R exposed cells became irregularly shrunken and rounded, with some becoming disintegrated. These exposed large areas with no cells present. P188 (predominantly 100 μmol/L - 1 mmol/L), delivered at the start of the reoxygenation, provided the most apparent protection of cellular morphology, with more cells appearing to have normal morphology and fewer cells showing irregular shrinking and rounding. The presence of P188 in C/N conditions did not significantly change the overall appearance of the cells. When PEG was used instead of P188, no improvement in cellular morphology was observed.

4 | DISCUSSION

Various I/R injury models have been used to show that P188 is cell-protective when administered before and/or during I/R;[3,29-35] yet, its exact mechanism of protection is not well understood. Considering that during reperfusion, cardiomyocytes, and other cell types, continue to become less viable and die until their environment stabilizes,

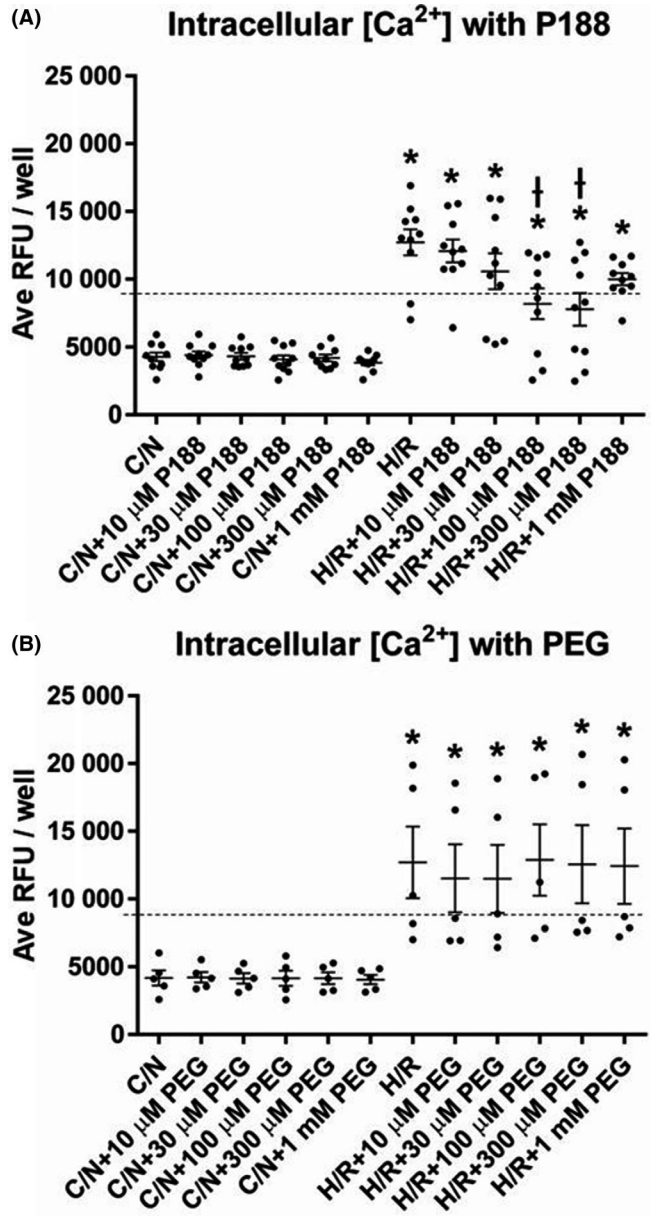

FIGURE 6 Measurement of $[Ca^{2+}]_i$. (A) The significant increase in $[Ca^{2+}]_i$ during H/R compared to cells under C/N conditions was significantly decreased with P188 concentrations of 100 μmol/L and 300 μmol/L. The dashed line represents the average hypoxia only level of ~9200 RFU/well. No significant effect of P188 was observed under C/N conditions. Data expressed as dot plots of individual data points together with the average RFU/well ± SEM bars; N = 10, 4-6 replicate wells per treatment per experiment, *vs C/N, ł vs H/R media only. (B) No significant effect of PEG on $[Ca^{2+}]_i$ was observed under C/N or H/R conditions. Data expressed as dot plots of individual data points together with the average RFU/well ± SEM bars, N = 5, 4-6 replicate wells per treatment per experiment, *vs C/N

this clinically more relevant time point requires more research. Unfortunately, few studies have examined the effect of P188 when given only during reperfusion, so even less is known about this aspect of its mechanism of action and whether the mechanisms have any similarities.

As clinicians, we focus mainly on examining the effect of P188 when administered during reperfusion. In a pig model of ST-segment elevation MI (STEMI), induced by 45 minutes of endovascular coronary occlusion, we have shown that 250 mg/kg P188 given during the 4-hour reperfusion led to a significant decrease in infarct size and protection of mitochondrial function.[36] Rat isolated hearts receiving 1 mmol/L P188 during the 2 hours of reperfusion following 30 minutes of global ischemia had sustained improvement of coronary and myocardial function, and decreased infarct size.[37] However, to best determine the protective mechanism of action of P188 when given only upon reperfusion, in-vitro studies using the various cell types present in a functioning heart are required. Since cardiomyocytes play a key role in the heart, we chose an in-vitro model of confluent isolated adult mouse cardiomyocytes designed to better mimic the status of these cells in *ex-* and in-vivo models. The cells were then subjected to an H/R protocol to simulate I/R. This protocol caused significant damage to the cells, including increased $[Ca^{2+}]_i$ and altered morphology, especially upon reoxygenation following the hypoxia, when compared to cells under C/N conditions.

In this study, P188 treatment during reoxygenation produced a dose-dependent attenuation of cellular dysfunction parameters caused by reoxygenation after hypoxia, with optimal effects observed at concentrations of 100-300 μmol/L P188. In fact, concentrations of 30 μmol/L - 1 mmol/L P188 improved cell number/viability to levels above that of hypoxia only, that is, it completely abolished the reoxygenation injury. Improved cell number/viability with P188 present during reoxygenation was also observed with assays measuring dehydrogenase enzyme function and caspase-3 activity. Additionally, the P188 concentrations of 100 μmol/L - 1 mmol/L present during reoxygenation significantly decreased cell membrane damage after H/R as assessed by two different methods, abolishing the effect of reoxygenation injury and indicating cell membrane repair. In most of the experiments performed, reoxygenation caused a significant increase in $[Ca^{2+}]_i$, which was substantially reduced in the presence of 100 μmol/L and 300 μmol/L P188. These concentrations also brought $[Ca^{2+}]_i$ to below that caused by hypoxia only, completely abolishing the effect of reoxygenation injury again in the majority of experiments. When P188 was present during the reoxygenation period, fewer cells appeared shrunken and rounded, with the majority showing normal morphology as observed in the C/N groups. This indicates that P188 can also play a role in maintaining functional cell morphology.

When copolymers like P188 are in solution, they exist as a dynamic population of grouped micelles and single molecules. It has been proposed that the single molecules of P188 in solution are most likely to confer protection compared to grouped P188 micelles because the molecule's hydrophobic portion is more readily available for insertion into, and interaction with, the damaged section of the membrane, as indicated by X-ray scattering, atomic force microscopy, and computer simulations.[16-18] It is possible that there were insufficient individual molecules available to provide adequate protection in the assessed cellular parameters when lower concentrations of P188 (eg, 10 μmol/L, 30 μmol/L) were used. With 1 mmol/L

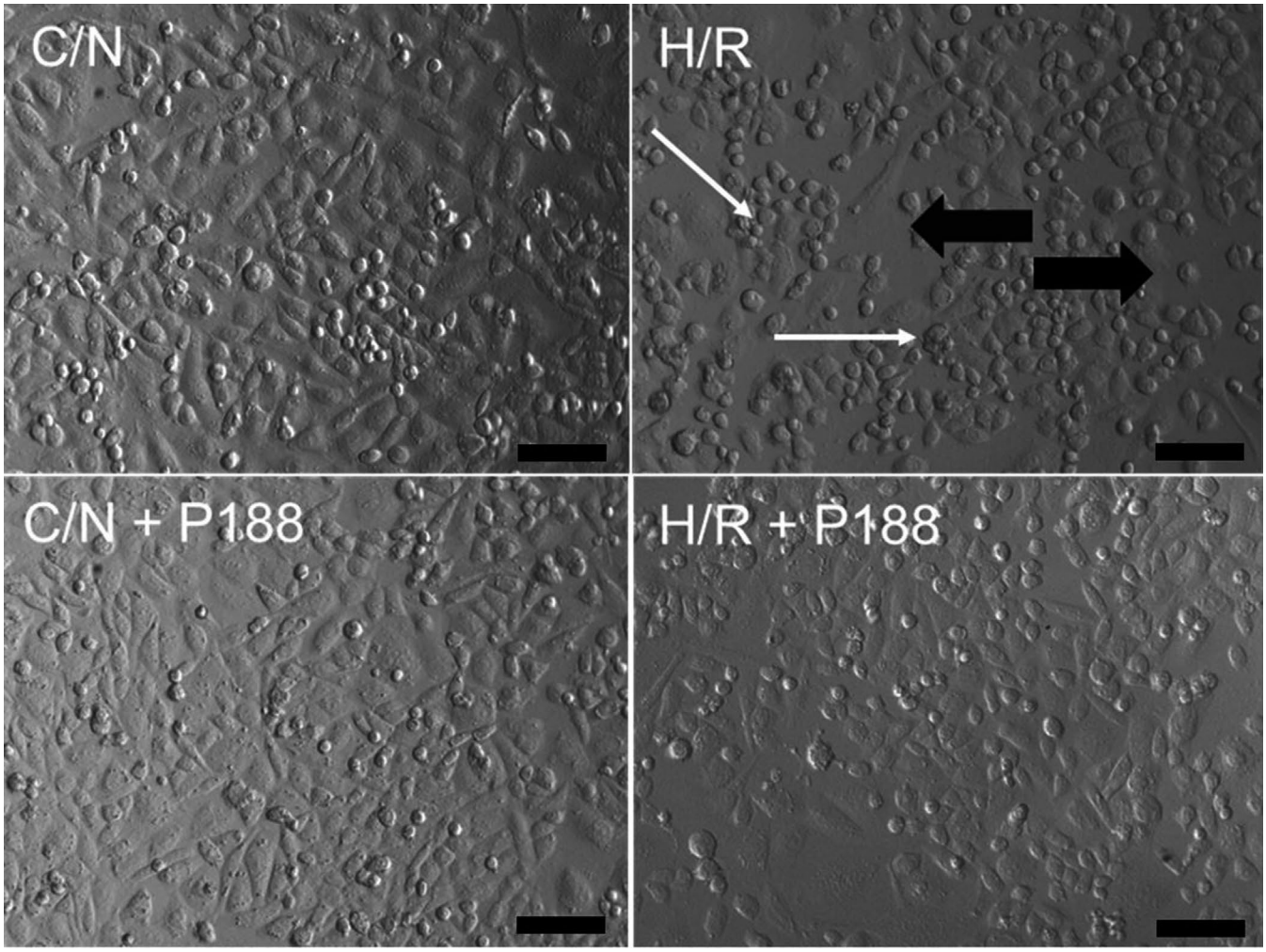

FIGURE 7 Cellular morphology. Representative photos of each group (C/N ± P188, H/R ± P188) show what was consistently observed in the experiments conducted. C/N group shows the normal condition and morphology of the cells at the end of the experiment, and the addition of P188 during reoxygenation did not significantly change the appearance of cells in C/N conditions. The H/R group shows large areas with no cells present (large black arrows), irregularly shrunk and rounded cells, as well as disintegrated cells (small white arrows); addition of P188 during reoxygenation prevented the majority of cells from shrinking and rounding, and most cells appeared to have nearly normal morphology. Cells that underwent H/R and given PEG during reoxygenation appeared the same as the H/R only group shown in the figure. Scale bars are 100 μm in length

P188, increased micelle formation may have been the cause of its lower protective ability.[16]

Under C/N conditions, where no measurable cellular injury occurred as assessed by our assays, no beneficial, or adverse, effect of any P188 concentration was observed. This may be because there were no detectable damaged sections of the membrane for the hydrophobic portion of P188 to interact with.

In our experiments with PEG (MW 8000 g/mol), a completely hydrophilic molecule with a similar MW to P188, concentrations of 10 μmol/L - 1 mmol/L PEG administered during reoxygenation had no protective, or adverse, effect in both C/N and H/R conditions. Several other studies using this same PEG compared to P188 have shown the same lack of a protective effect. In the Bartos et al study,[36] P188 infused immediately upon reperfusion following a STEMI reduced infarct size and preserved mitochondrial integrity and function; the same dose of PEG provided no protective benefit. In-vitro studies using C2C12 myoblasts exposed to a hypo-osmotic stress/isotonic recovery insult showed similar results. Concentrations of P188 from 14 μmol/L to 150 μmol/L were protective when added during the isotonic recovery based on assessments of LDH release; however, comparable concentrations of PEG added during isotonic recovery had no effect on decreasing the release of LDH from these myoblasts.[21,38] This may be because PEG does not contain a hydrophobic portion, which is proposed to be the component of copolymers needed to insert into, and interact with, the damaged sections of a cell membrane.

The cell membrane is composed of a double layer of various lipids and proteins that work together to maintain the cell structure and function. Lipid rafts are specialized sub-compartments of the cell membrane made up of sphingolipids, cholesterol, and a variety of proteins that regulate a number of signaling processes.[39] Several types of stressors, such as oxidative stress occurring during I/R, can

lead to the disruption of lipid rafts, which exposes the cell to even higher $[Ca^{2+}]_i$ and apoptotic signaling,[39-42] as was observed in our experiments. With the hydrophobic portion of the lipid raft exposed due to this stress disruption, we imagine the hydrophobic segment of a P188 molecule can interact through hydrophobic interactions and insert into the cell membrane at this location. This then stabilizes the integrity of the membrane and allows for normal cellular function. P188 had no effect on cells under C/N conditions because of little to no disruption of lipid rafts, and PEG has no hydrophobic component. Direct assessment of lipid raft disruption and repair was outside the scope of this study.

Interestingly, it has been postulated that increasing the length of hydrophilic PEO chains and/or the concentrations used may increase the protective ability of PEG, most likely through increased interactions with more lipid head groups and/or other cell membrane components, such as liposomes and proteins.[21,43] Proving this potential for higher molecular weight PEG to be cardioprotective, studies have used PEG (MW 15 000-20 000 g/mol) in isolated cardiomyocytes exposed to H/R and rat models of MI, cardiac arrest, and hemorrhagic shock. These in-vitro studies showed that this high MW PEG significantly inhibited H/R-induced apoptosis and decreased lipid raft coalescence, another indicator of cell membrane injury.[41,42] The in-vivo studies using rat models of MI, cardiac arrest, and shock showed improved microcirculation, overall cardiac function, and post-resuscitation survival, as well as decreased infarct sizes and markers of apoptosis.[44-49] Based on this information, the PEG (MW 8000 g/mol) used in our experiments most likely did not have enough hydrophilic PEO chains, or was not used at a high enough concentration, to interact with the damaged membranes in our model.

The present study does have natural limitations, such as the use of only an in-vitro model of cardiomyocytes to mimic I/R injury and assess the cellular protection capability of P188, and the measurement of a limited number of indices to assess cellular function and membrane integrity. Although the cells used in this study express a number of cardiomyocyte markers, contain abundant mitochondria, and possess contractile proteins, they are unable to contract due to disrupted sarcomeres. This lack of contraction/relaxation most likely decreased our model's injury response to H/R, leading us to use a greater and longer hypoxic insult than what likely would be needed with cultures of beating cardiomyocytes. Another circumstance that possibly decreased the cells' H/R injury response was the confluency of the cultures. The cardiomyocytes were allowed to grow into confluent cultures to better mimic the normal in-vivo status of these cells in the heart, allowing cell contacts and intracellular signaling to occur. Thus, our model did provide more insight into the effect of H/R on confluent cultures of cardiomyocytes and the protection provided to these cells by P188 when it is administered at the start of reoxygenation. However, with in-vitro cultures of one cell type, there is none of the different cell-cell interaction and organization that is found in an intact organ or tissue, which could have the possibility to compensate in abnormal, stressful situations, making straight extrapolation of in-vitro outcomes to ex-vivo and in-vivo models difficult.

Our study only measured a limited number of indices to assess cellular viability and membrane integrity; therefore, we tried to ensure our conclusions for the different outcome measures using endpoint assay techniques that complemented each other. In addition to the CyQUANT assay that assesses cell number/viability through the fluorescent measurement of DNA content only in healthy cells, the Promega MTS assay was used in a subset of experiments to measure the amount of formazan product produced by the dehydrogenase enzymes in metabolically active cells. To assess the viability of these cells another way, in relation to apoptotic cell death, another subset of experiments was used to measure caspase-3 activity, which increases in association with apoptotic processes. The photos taken of the different treatment groups added another dimension to the assessment of cell number/viability.

Cell membrane injury/repair was assessed two different ways; measurement of the intracellular enzyme LDH released from damaged cells into the culture media, as well as measurement of the amount of the styryl dye FM1-43, which remains extracellular unless membrane damage allows it to become incorporated into the lipid bilayer where it then fluoresces. Assessment of $[Ca^{2+}]_i$ was also conducted, since Ca^{2+} is an essential signaling molecule in cardiomyocytes. Changes in $[Ca^{2+}]_i$ can be caused by a number of factors, including membrane damage, and can be used to assess cellular viability and function.

Further measurements of cell viability and function, such as changes in other cell signaling molecules, other cell death markers, and mitochondrial function, as well as other assessment of membrane integrity, including lipid rafts as discussed earlier, would have offered more information toward defining the mechanisms of cellular protections by P188 when it is administered only at the start of reoxygenation to attenuate H/R injury. Additional studies incorporating these complementary assessments should be undertaken in the future.

The risk of bias is inherent in many experimental studies, as it can be difficult to exclude every possible category of bias. In our study, the researchers were not blinded to the experimental treatment groups, nor to the outcome assessments. Additionally, we expected P188 to have some degree of cellular protection due to its positive performance detected in other in-vitro studies already published in the literature.[21-23] These forms of bias, such as performance and detection, can possibly skew data toward a more positive, favorable outcome. To counteract these possible biases, we performed a number of measures to both decrease bias and strengthen our study outcomes. Selection bias was minimized using several different lots of cardiomyocytes and a number of different passages of the cells, 96-well plates of confluent cells were randomized to either the C/N or H/R experimental exposure, and the location of different treatment well groups was varied across the experimental plates, all while experimental conditions were kept as identical as possible. Attrition and reporting bias were eliminated using all the wells in an experimental treatment group to calculate the average for that group in each experiment. This led to larger standard errors in one or two treatment groups for several experiments, but had no impact on the

final cumulative data since averages of each treatment group for each experiment were used to display the data and perform statistical analyses.

Even though in-vitro studies have numerous intrinsic limitations, their strength lies in the fact that they can provide essential information into the mechanisms of injuries, as well as therapies, on specific cells and cellular properties. The results of our study using confluent cultures of cardiomyocytes suggest that P188 may also be able to protect other isolated cell types (eg, endothelial cell, neurons) in similar ways, when the cells are affected by an injury, such as H/R, that disrupts cell membrane integrity. And, the protection of a cell type, whether cardiomyocytes, endothelial cells, or neurons, may then lead to organ protection. Therefore, studies need to be conducted in other cell types, and more importantly, in ex- and in-vivo models, to validate the results of this study, as well as gain more information about P188's mechanism of action when administered only during reperfusion, which could lead to P188 being repurposed as a therapy to abolish reperfusion injury in the future.

In summary, our results demonstrate that (a) reoxygenation potentiates the injury caused by hypoxia alone in our in-vitro mouse cardiomyocytes model of simulated I/R; (b) P188, at the concentrations used in these experiments and administered at the beginning of the clinically relevant time point of reoxygenation, can protect isolated cardiomyocytes from reoxygenation injury; and (c) the hydrophobic component of the P188 molecule is needed to stabilize damaged cell membranes and protect cardiomyocytes from reoxygenation injury. P188's protective effect is likely due to its unique chemical properties; the hydrophobic central portion inserts into the damaged section of the cell membrane and, through hydrophobic interactions, anchors in place, while the hydrophilic ends interact with the lipid head groups of the external membrane surface, stabilizing both the molecule and membrane, and ultimately restoring cellular integrity and function. These findings add to the growing body of literature demonstrating the protective role P188 has against I/R injury, and improves on the understanding of the molecule's mechanism of action, especially when only administered during reperfusion.

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DISCLOSURE

None of the authors has any potential conflicts of interest.

AUTHOR CONTRIBUTIONS

MMS and MLR participated in research design; MMS conducted experiments; MMS and MLR performed data analysis; MMS, JAB, DY, and MLR wrote or contributed to writing the manuscript.

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