M22 HORMESIS IN MOUSE EMBRYONIC STEM CELL MODELS

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Abstract

Cryoprotectant toxicity (CT), or the injury due to cryoprotectant (CPA) exposure, remains the most limiting barrier to performing successful organ cryopreservation. An intervention alleviating the stress induced by CT in mouse embryonic stem cell (mESC) models may translate well into tissue and organ models of cryopreservation. Such discoveries could enable large-scale organ banking and, potentially, end the transplantable-organ shortage. In this study, I sought to see if CT could be combated through a hormetic approach. Hormesis will be defined as the negation of the detrimental effects of a high, lethal dosage of a stressor via a prior nonlethal dosage of the same stressor with a recovery period in between each exposure. Wild-type mESCs were exposed to a pretreatment of 0%, 1% or 2% CPA (M22 in these studies) in embryonic stem cell medium (ESCM), allowed to recover, and subsequently exposed to a lethal dosage of 9% M22 in ESCM. Pretreatments of 1% and 2% M22 in ESCM resulted in greater loss of viability as compared to no previous exposure to M22. This study failed to induce a hormetic effect in mESCs, however, hormesis remains a plausible mechanistic approach to the alleviation of CT due to CPA exposure. More research exploring a broader selection of M22 pretreatment conditions and recovery periods need to be observed using experimental protocols similar to those outlined in this paper.

1. Introduction

Cryopreservation is a toxic process to biological systems due to cold exposure, cooling and warming rates, ice formation, and predominantly, cryoprotectant toxicity (CT), amongst others. Finding methods to alleviate these cryoinjuries has become the central goal of cryobiologists (Schumacher et al., 2019). If organs could successfully be cryopreserved and banked, millions of lives would be saved due to successful organ transplants becoming more frequent and feasible. The cryopreservation of rabbit kidneys has successfully been achieved through vitrification using a combination of cryoprotective agents (CPAs) (Fahy and Ali, 1997). However, due to the high toxicity of CPAs, cryopreserved organs are not viable options for human organ transplantation thus far. Using wild-type mouse embryonic stem cells (mESC) as our model systems, an interventions associated with hormesis is sought to make organ cryopreservation a viable solution to the growing transplantable-organ need.

Hormesis is an adaptive response achieved through an overcompensation-induced stimulation. A low level of stress is administered prior to a higher, more enhanced level of stress. Through this biphasic dose exposure there is generally a 30-60% increase in viability, as compared to controls (Calabrese and Baldwin, 2002). My lab generated mutant strains of mESCs that are resistant to low levels of M22, a CPA of organ vitrification solution created by our lab's collaborator, Dr. Greg Fahy. The mutant strains of mESC were screened and selected after a seven-day exposure to 9% M22 in embryonic stem cell medium (ESCM) at 37°C (Cypser et al., 2019). Each strain was found to have increased growth in viability after this treatment with M22 as compared to controls, and the strains' unique mutations each unequivocally conferred their resistance. In another study detailed in the same manuscript, mESCs were exposed to 60% M22 at 2°C for 8 hours. Within 4 hours of exposure, only 30% of mESCs remained viable and

continued to survive for 8 additional hours, potentially due to the cells undergoing resistant physiological changes. Using the repeatable pattern of CT resistance, a hormetic effect could be induced to alleviate CT in mESC.

Hormetic effects have been documented during the exposure of an initial stressor that is one fourth of the lethal dosage, then followed with the exposure of the lethal dosage of that same stressor (Cypser et al., 2006). By studying the lethal dosages for mESCs identified in the two studies listed prior and performing preliminary studies, an experimental protocol was generated in efforts to test if exposing wild-type mESCs to a cryotoxic stressor, M22, induces a resistant phenotype to subsequent cryobiological exposures after a recovery period through the process of hormesis. If mESCs experience increased viability through this hormetic approach as compared to controls, then hormesis could be a novel intervention for alleviating toxicity due to CPA exposure and could be tested in mammalian organ models by our collaborators, with the ultimate goal of implementing an intervention in human organ cryopreservation.

2. Background

Organ cryopreservation is performed at cryogenic temperatures (below -100°C). These low temperatures terminate all enzymatic and chemical activities inside of cells, tissues, and organs (Lemaster and Oliver, 1995). However, due to cellular osmolarities, they also result in the formation of ice crystals that cause irreversible damage to cellular membranes and ultimately lead to cells effectively bursting. In efforts to combat this issue, organ cryopreservation is performed through vitrification, the process by which high molarities of CPAs are used to prevent liquids, primarily water, from crystalizing and instead become less and less viscous and ultimately acquire an amorphous form in cryogenic temperatures (Fahy at el., 2006). Though ice crystals are prevented, the high molarities of CPA exposure create a toxic environment for the cells and often lead to the loss of function and viability (Best, 2015).

M22 is a vitrification solution that is optimized for organ cryopreservation. In a study using rabbit kidneys, it was shown that when perfused for 25 minutes at -22°C to a concentration of 9.4 M M22 and then washed out while thawing, rabbit kidneys were able to make a complete functional recovery. With this procedure, CT and osmotic stress remain an obstacle (Fahy et al., 2004). Finding protocols that optimize the usage of M22 as a CPA for vitrification is fundamental for the progression of organ cryopreservation.

A semi-automated method of the loading and unloading of 100% M22 has been designed using precision-cut liver slides (PCLS) (Guan et al., 2012). In this study, PCLS were gradually exposed to M22 and subsequently incubated for 3 hours at 37°C in efforts to develop a method that maximized viability and minimized osmotic stress and CT. PLCS showed a high tolerance for osmotic changes and thus were predicted to be mainly affected by CT over osmotic stress. A possible method for the alleviation of osmotic stress was presented in this study, and these

findings have been confirmed in mESCs (Schumacher et al., 2019); however, loss of viability due to CT remains the most deleterious obstacle. Many different studies have been designed to target this issue, each exploring different methods of application of CPAs. In this paper, a new intervention for CT is postulated.

Hormesis is a protective mechanism that modifies cellular and molecular pathways to extend homeostatic ranges in response to oxidative and chemical stressors. During cellular stress, the expression of stress-resistant genes is upregulated, thus enhancing the production of a range of protective responses, such as the production of heat-shock proteins, protein chaperones, anti-oxidative enzymes, growth factors and cytokines (Mattson, 2008). Hormesis has been shown to activate protective pathways against cell, tissue, and organ toxicity (Zhang et al., 2008). Due to this, and in efforts to successfully perform organ cryopreservation through vitrification, it is important to consider hormesis as a plausible mechanistic approach.

Preliminary experiments were performed to identify possible pretreatment conditions and the lethal concentration of M22 on mESC that could induce a hormetic effect. By observing previous studies, concentrations of 0.5, 1, 2, 4, and 8% M22 in ESCM for 0, 1, 2, and 4 hours were selected. At all durations and concentrations of M22 in ESCM, except for any duration of 0.5% M22 in ESCM, morphological changes were observed on the mESC, with 8% M22 being the most deleterious. The lethal dosage of 9% M22 in ESCM was selected for this study due to being thoroughly examined during the generation of mutant strains of mESC (Cypser et al., 2019) and in efforts to ensure detrimental conditions would be met for mESC. The pretreatments of 1% and 2% M22 in ESCM for one hour were selected due to inducing the most minimal morphological changes and closely representing one fourth of the lethal dosage.

3. Materials and Methods

3.1 Reagents and solutions

M22 is a complex, eight-component vitrification solution developed by 21st Century Medicine (Fontana, CA). M22 functions as a penetrating CPA to rabbit kidneys at a concentration of 9.4 M (Fahy et al., 2004). M22 is composed of 2.8 M Me2SO, 2.8 M formamide, 2.7 M ethylene glycol, 0.5 M N-methylformamide, 0.3 M 3-methoxy-1,2-propanediol, 2.8% w/v (less than 0.006 M) polyvinylpyrrolidone, 1% w/v (less than 0.006 M) polyvinyl alcohol-polyvinyl acetate copolymer, and 2% w/v (less than 0.03 M) polyglycerol (Fahy et al., 2004; Fahy and Wowk, 2014). I refer to "% M22" as the percent of this working concentration used.

DPBS (1X Dulbecco's phosphate-buffered saline supplemented with calcium and magnesium; Gibco, product 14040-133) is used to rinse cells of old medium and cellular wastes before dissociation or given fresh medium (Chick et al., 2014).

1X TrypLE Express with phenol red and EDTA (Gibco, product 12605-028) is an animal-origin-free replacement for porcine trypsin and was solely used for dissociation of mESC.

Embryonic stem cell medium (ESCM), optimized for mESC, is KnockOut Dulbecco's Modified Eagle's Medium (Gibco, product 10829-018) supplemented with 15% fetal bovine serum (Tissue Culture Biologicals, product 104), Glutamax (2 mM L-alanyl-L-glutamine dipeptide in 0.85% NaCl; Gibco, product 35050-061), 0.1 mM non-essential amino acids (Gibco, product 1140-050), 25 units per ml of penicillin-streptomycin (Thermo Fisher, product 15140122), 55 μM beta-mercaptoethanol (Thermo Fisher, product 21985-023), and 1,000 unit

per ml of leukemia inhibitory factor (AMSBio, product AMS-263-100). (Chick et al., 2014). ESCM and LM5 are isotonic solutions (Meryman and Douglas, 1982).

MTT solution is 5 mg of tetrazolin salt per ml of MTT reagent (Thiazolyl Blue Tetrazolium Bromide; Research Products International, product M92050) dissolved in KnockOut Dulbecco's Modified Eagle's Medium. Solubilization solution is acidic isopropanol (0.1N HCl).

mESC were generated from a F1 hybrid background (C57BL/6J x 129X1/SvJ). Each experiment cells from a different passage or thawed stock of cells, all being less than passage twelve. For all experiments, cells were thawed and cultured under standard cell culture conditions prior to harvesting for experimentation (ESCM, at 37°C, 5% CO2) (Chick et al., 2009). Cells were seeded and proliferated in 12.5-cm^2 and then transferred into 75-cm^2 tissue-treated culture flasks when sufficient cell numbers were obtained. All procedures discussed in this paper were performed under aseptic conditions.

3.2 Growing and Seeding Cells

mESC were cultured under standard growth conditions in tissue-treated culture flasks. Cells were provided fresh ESCM approximately every 24 hours. First, visual examinations were performed to ensure no morphological changes, differentiations, or abnormalities had occurred. Then flasks were aspirated of supernants and had DPBS added for 5 minutes. The remaining supernatants and DPBS were then aspirated and fresh ESCM was provided (Chick et al., 2009).

In preparation for the experimental seeding of the cells, the supernatants in a tissue-treated culture flask were aspirated, then given 1X TrypLE and incubated for 10 minutes to dissociate the cells from the flask. After 10 minutes, an equal amount of ESCM was added to neutralize the activated 1X TrypLE. All components in the flask were thoroughly mixed and

collected into a 50 mL centrifuge tube and centrifuged (200 RCF, 5 minutes, 25°C, ACC/DEC 4). The centrifuge tubes were aspirated of supernatants and the cell suspension was resuspended in ESCM. Cells were counted manually using a hemocytometer (average of 5 counts were used) and a microscope.

3.3 Pretreatment

The ESCM suspensions were aliquoted into three different 75-cm^2 tissue-treated culture flasks, each containing 2 million cells. All flasks were incubated to allow for cellular adherence. After 24 hours, the flasks were aspirated of supernatants, rinsed with DPBS, and exposed to their designated pretreatment group: 0%, 1% or 2% M22 in ESCM. All flasks were placed in an incubator at 37°C for 90 minutes, after which all were rinsed with DPBS, provided fresh ESCM and placed in the incubator for a 3-hour recovery period.

3.4 Challenge

The cells in each flask were dissociated using 1X TrypLE, counted, and aliquoted into every well to be used into 96-well plates in triplicate. The three plates were labeled accordingly to the time at which the plate would be analyzed: 0 hour, 12 hour and 24 hour. Each well per plate that contained cells was then exposed to the lethal dosage of 9% M22 in ESCM, this is termed the challenge of the experiment. ESCM was then added to empty wells on each plate to be used as phenol red standards to control for absorbance due to the phenol red contained in the ESCM. The outer wells were unused to avoid edge effects.

3.5 Methyl thiazolyl tetrazolium (MTT) colorimetric assay

The MTT colorimetric assay measures cellular mitochondrial activity (Tominaga et al., 1999). Mitochondrial activity is correlated to the number of viable cells in a given sample. First, all plates were incubated for their assigned challenge duration, after which each well per plate containing cells or phenol red standards was given 20 µl of MTT, placed on a shaker (setting 6, 30 seconds) and returned to the incubator for three hours at 37°C. Through cellular reactions that are not yet understood, cells convert MTT into formazan, a water-insoluble crystal (Hördegen at el., 2006). Upon completing the incubation time, 100 µl of isopropanol, a solubilization solution, was added to each well containing cells or phenol red standards and shaken (30 minutes protected from light). Visual inspections of every well under a microscope ensured all formazan crystals were solubilized. Absorbance through each well was measured via an Epoch plate reader at 570 nm, with background absorbance subtracted at 690 nm, within fifty minutes of the addition of isopropanol. The mean absorbance value for the phenol red standards was subtracted from the initial absorbance value to create a final absorbance value for every sample (Mosmann, 1983; Tada et al., 1986).

3.6 Analytics

Two-way ANOVA's and Tukey's HSD test's were performed in R Studio (Version 1.1453). Figures and tables were generated in Microsoft Excel. Absolute values were normalized within each pretreatment to the corresponding pretreatment's 0-hour group to account for cell counting and proliferation variability. Reported measurements are means ± standard errors of the means.

4. Results

Data from three independent experiments was gathered in attempt to interpret the effect of M22 exposure through a hormetic approach on mESC viability. The pretreatment and challenge duration were significant factors to mESC viability in all experiments, except for Experiment 3 (Table 2). The pretreatment and challenge duration in combination were also significant factors to mESC viability in all experiments, except for Experiment 1 (Table 2). Further analyses were performed to compare individual pretreatments and the challenge duration to the control for that same challenge duration.

Experiment 1 results show viability was lower in the pretreatment of 1% M22 in ESCM during a 24-hour challenge duration when compared to the control pretreatment of 0% M22 in ESCM ($p=0.023$; Fig. 1). In Experiment 2, the viability was lower in the pretreatment of 2% M22 in ESCM during a 12- and 24-hour challenge duration when compared to the control pretreatment of 0% M22 in ESCM ($p=0.001$ and $p=0.009$, respectively; Fig. 2). In Experiment 3, viability was lower in the pretreatment of 1% M22 in ESCM during a 12-hour challenge duration when compared to the control pretreatment of 0% M22 in ESCM ($p=0.011$; Fig. 3).

In efforts to increase the statistical power a summary of all three experiments was generated. The summary results show that viability was lower in the pretreatment of 1% M22 in ESCM during a 12-hour challenge duration when compared to the control pretreatment of 0% M22 in ESCM ($p=0.001$; Fig. 4). Viability was also lower in the pretreatment of 2% M22 in ESCM during a 12-hour challenge duration when compared to the control pretreatment of 0% M22 in ESCM ($p=0.047$; Fig. 4). All other experimental conditions showed no viability difference between the pretreatment groups when compared to the control.

5. Discussion

This study aimed to induce a resistant phenotype to the cryotoxic stressor M22 in wild-type mESCs through the process of hormesis in an effort to find a new intervention for alleviating CT. The pretreatment of 1% and 2% M22 in ESCM caused a loss of viability when compared to the control throughout the 12-hour challenge duration of 9% M22 in ESCM. These results indicate that the pretreatments, in combination with their recovery periods, failed to induce a hormetic effect and thus the exposure to the challenge duration was more detrimental on the mESCs. These results assert the necessity of exploring more pretreatment conditions and recovery periods.

Preliminary experiments were performed in efforts to predict the pretreatment conditions and the challenge lethal dosage that would induce a hormetic effect. The recovery period was not a factor observed in these studies. Therefore, it could arguably have caused the hormetic effects on mESCs to diminish or have not allowed for appropriate cellular recuperation. In a study observing radiation hormesis, a dosage of 0.2-Gy x-irradiation was administered to mice every other day. Functional and phenotypic adaptations to the radiation were suggested to have occurred during the recovery period (Pickrell, 2009). To determine the importance of the recovery period in regards to the induction of a hormetic effect on mESC, observations of longer and shorter durations of the recovery period are necessary.

The lethal dosage at all durations of the challenge resulted in a drastic loss of viability for all pretreatment conditions, thus, suggesting it to adequately represent the challenge needed for the induction of a hormetic effect on mESC. Though the pretreatments caused decreases in viability, they could have failed to sufficiently stress the mESCs and induce a CT resistant phenotype. Observations of pretreatments conditions that more closely resemble one fourth of

the lethal dosage are necessary for the induction of a hormetic effect. Higher concentrations of M22 and longer pretreatment durations need to be explored. Future studies should also consider the effects of multiple pretreatment exposures.

As a result of the administered periodical radiation exposure in the radiation hormesis study mentioned prior, the production of lymphocytes decreased after 10 days of exposure but increased after 28 days (Pickrell, 2009). Multiple pretreatment exposures were not explored in this paper but could be critical for the induction of a hormetic effect. Though the study outlined in this paper failed to generate a resistant phenotype through the process of hormesis, more research and mechanistic approaches are needed before the exposure of M22 on mESC is considered non-hormetic.

Culturing mESC is a time and resource consuming process. The experimental protocol presented here maximized the statistical data collected per experiment. This allowed for multiple pretreatment conditions to be analyzed and a variety of challenge durations to be observed independently of one another. Through this systematic approach, a new experimental setup was presented along with the possibility that the solution to CT due to CPA exposure could be through a hormetic approach. Hormesis could lead to significant interventions within organ cryopreservation and other toxic events.

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TABLES

Table 1. Experimental results

	Pretreatment (% M22)	Challenge Duration (hours)	Absolute Absorbance Mean	Absolute Absorbance SEM	Absolute Absorbance p vs con	Normalized Viability Mean	Normalized Viability SEM	Normalized Viability p vs con	n
Experiment 1	0 (Control)	0	0.191	0.012	-	100.00	0.00	-	6
		12	0.141	0.009	-	75.00	6.26	-	6
		24	0.078	0.007	-	41.96	5.55	-	6
	1	0	0.178	0.006	0.953	100.00	0.00	1.000	6
		12	0.108	0.004	0.082	61.07	3.92	0.236	6
		24	0.040	0.005	0.032	22.41	2.33	0.023	6
	2	0	0.204	0.010	0.955	100.00	0.00	1.000	6
		12	0.147	0.007	1.000	73.46	6.36	1.000	6
		24	0.052	0.004	0.351	25.71	1.72	0.098	6
Experiment 2	0 (Control)	0	0.348	0.008	-	100.00	0.00	-	8
		12	0.231	0.011	-	66.92	4.02	-	8
		24	0.155	0.009	-	44.86	2.98	-	8
	1	0	0.309	0.004	0.026	100.00	0.00	1.000	8
		12	0.181	0.006	0.001	58.45	1.91	0.133	8
		24	0.124	0.005	0.137	39.96	1.14	0.788	8
	2	0	0.322	0.013	0.330	100.00	0.00	1.000	8
		12	0.170	0.005	0.000	53.50	2.60	0.001	8
		24	0.106	0.005	0.002	33.41	2.04	0.009	8
Experiment 3	0 (Control)	0	0.194	0.010	-	100.00	0.00	-	4
		12	0.105	0.015	-	54.21	7.07	-	4
		24	0.079	0.002	-	42.03	2.25	-	4
	1	0	0.203	0.013	0.999	100.00	0.00	1.000	4
		12	0.071	0.006	0.253	36.66	3.63	0.011	4
		24	0.083	0.002	0.999	43.43	3.49	1.000	4
	2	0	0.252	0.013	0.001	100.00	0.00	1.000	4
		12	0.101	0.011	1.000	41.40	4.16	0.986	4
		24	0.090	0.002	0.997	36.92	2.25	0.977	4
Summary (All Data)	**0 (Control)**	**0**	**0.244**	**0.101**	**-**	**100.00**	**0.00**	**-**	**18**
		12	**0.159**	**0.015**	**-**	**65.38**	**5.78**	**-**	**18**
		24	**0.104**	**0.006**	**-**	**42.95**	**3.59**	**-**	**18**
	1	**0**	**0.230**	**0.008**	**0.998**	**100.00**	**0.00**	**1.000**	**18**
		12	**0.120**	**0.005**	**0.182**	**52.06**	**3.15**	**0.001**	**18**
		24	**0.083**	**0.004**	**0.972**	**35.27**	**3.49**	**0.787**	**18**
	2	**0**	**0.259**	**0.012**	**0.001**	**100.00**	**0.00**	**1.000**	**18**
		12	**0.139**	**0.008**	**0.949**	**56.12**	**4.37**	**0.047**	**18**
		24	**0.083**	**0.004**	**0.997**	**32.01**	**2.00**	**0.088**	**18**

Normalized and absolute means, standard errors of the means (SEM), p-values, and sample sizes (n) of all experiments. mESCs had a pretreatment of 0%, 1% or 2% M22 in ESCM for 90 minutes and a recovery period of three hours prior to the challenge. mESC were then seeded at a density of 20,000 cells per well for three identical 96-well plates. The plates were exposed to the challenge of 9% M22 in ESCM for a 0-, 12-, or 24-hour incubation period at 37°C. Each plate was assayed after their respective incubation periods. P-values of pretreatment comparisons with the control pretreatment generated from Tukey's HSD tests.

Table 2. Two-way ANOVA results

	Effect	Absolute p > F	Normalized p > F
Experiment 1	Pretreatment	5.84E-05	3.95E-03
	Challenge Duration	2.00E-16	2.00E-16
	Pretreatment * Challenge Duration	0.0867	0.0563
Experiment 2	Pretreatment	1.05E-09	6.07E-05
	Challenge Duration	2.00E-16	2.00E-16
	Pretreatment * Challenge Duration	0.167	0.0242
Experiment 3	Pretreatment	8.72E-04	6.28E-01
	Challenge Duration	2.00E-16	2.00E-16
	Pretreatment * Challenge Duration	8.78E-03	0.03214
Summary (All Data)	**Pretreatment**	**2.44E-02**	**4.70E-04**
	Challenge Duration	**2.00E-16**	**2.00E-16**
	Pretreatment * Challenge Duration	**2.06E-01**	**1.89E-02**

Normalized and absolute two-way ANOVA results. mESCs had a pretreatment of 0%, 1% or 2% M22 in ESCM for 90 minutes and a recovery period of three hours prior to the challenge. mESC were then seeded at a density of 20,000 cells per well for three identical 96-well plates. The plates were exposed to the challenge of 9% M22 in ESCM for a 0-, 12-, or 24-hour incubation period at 37°C. Each plate was assayed after their respective incubation periods. P-values generated with pretreatment and challenge duration as factors affecting viability. Challenge duration was the most significant factor affecting viability, independently from the pretreatment conditions.

FIGURES

Figure 1

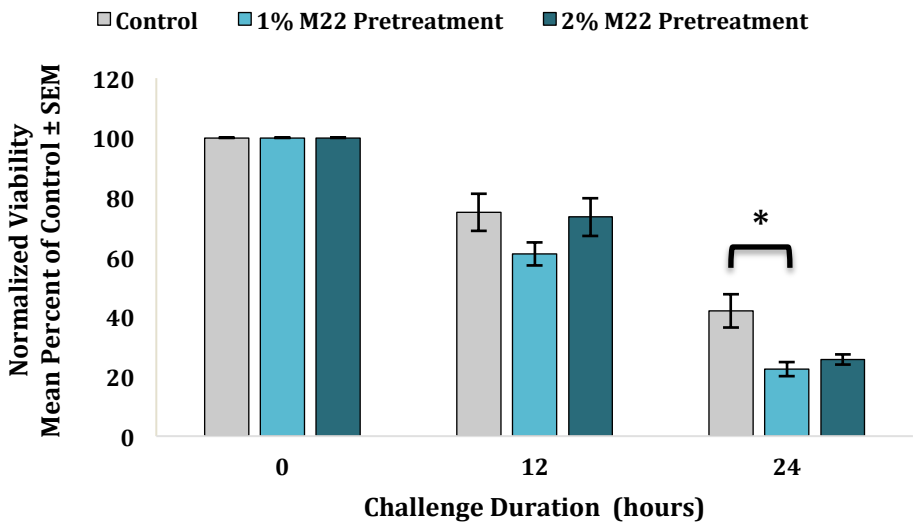

Fig. 1 Normalized viability results from Experiment 1. mESCs had a pretreatment of 0%, 1% or 2% M22 in ESCM for 90 minutes and a recovery period of three hours prior to the challenge. mESC were then seeded at a density of 20,000 cells per well for three identical 96-well plates. The plates were exposed to the challenge of 9% M22 in ESCM for a 0-, 12-, or 24-hour incubation period at 37°C. Each plate was assayed after their respective incubation periods. P-values generated from two-way analysis of variance with pretreatment and challenge duration as factors affecting viability. * $p < .05$, † $p < .01$, ‡ $p < .001$, § $p < .0001$.

Figure 2

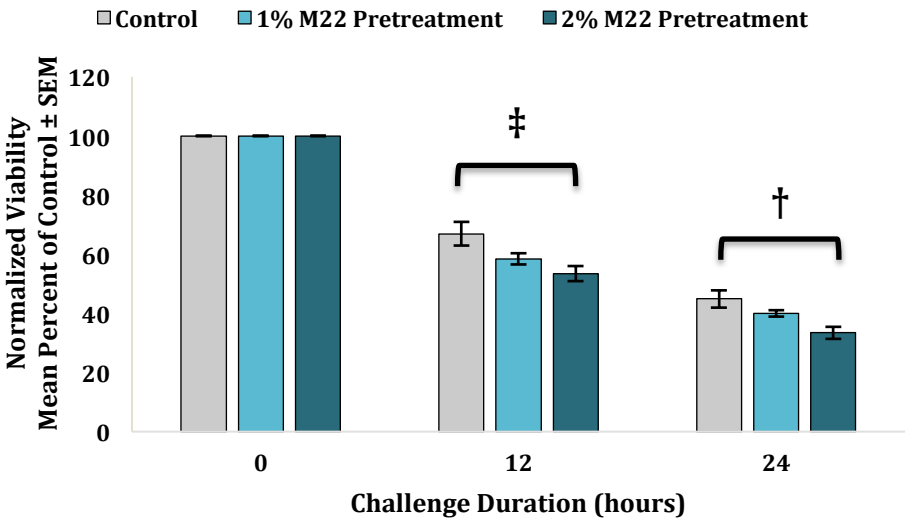

Fig. 2 Normalized viability results from Experiment 2. mESCs had a pretreatment of 0%, 1% or 2% M22 in ESCM for 90 minutes and a recovery period of three hours prior to the challenge. mESC were then seeded at a density of 20,000 cells per well for three identical 96-well plates. The plates were exposed to the challenge of 9% M22 in ESCM for a 0-, 12-, or 24-hour incubation period at 37°C. Each plate was assayed after their respective incubation periods. P-values generated from two-way analysis of variance with pretreatment and challenge duration as factors affecting viability. * $p < .05$, † $p < .01$, ‡ $p < .001$, § $p < .0001$.

Figure 3

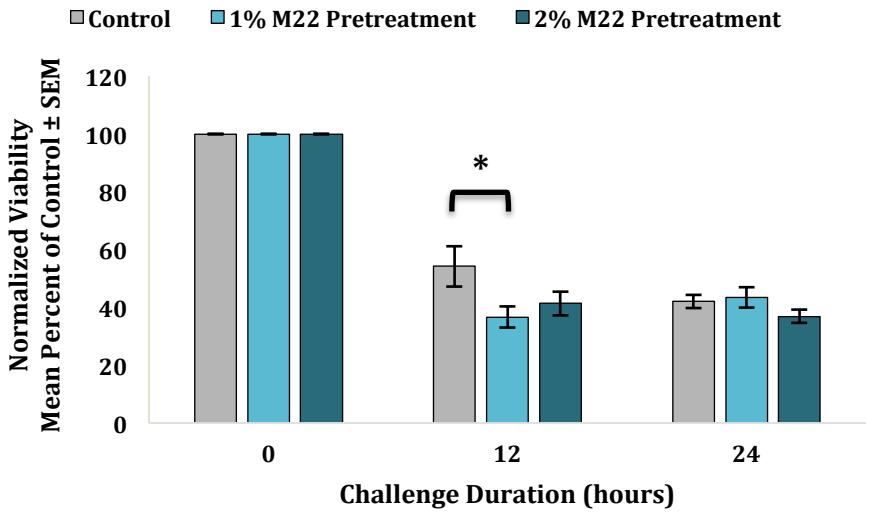

Fig. 3 Normalized viability results from Experiment 3. mESCs had a pretreatment of 0%, 1% or 2% M22 in ESCM for 90 minutes and a recovery period of three hours prior to the challenge. mESC were then seeded at a density of 20,000 cells per well for three identical 96-well plates. The plates were exposed to the challenge of 9% M22 in ESCM for a 0-, 12-, or 24-hour incubation period at 37°C. Each plate was assayed after their respective incubation periods. P-values generated from two-way analysis of variance with pretreatment and challenge duration as factors affecting viability. * $p < .05$, † $p < .01$, ‡ $p < .001$, § $p < .0001$.

Figure 4

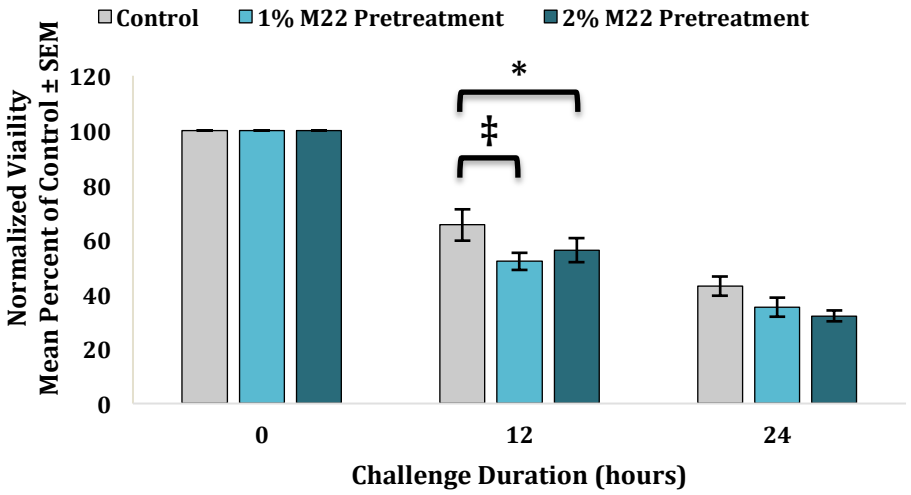

Fig. 4 Summary viability results from normalized data of all experiments. mESCs had a pretreatment of 0%, 1% or 2% M22 in ESCM for 90 minutes and a recovery period of three hours prior to the challenge. mESC were then seeded at a density of 20,000 cells per well for three identical 96-well plates. The plates were exposed to the challenge of 9% M22 in ESCM for a 0-, 12-, or 24-hour incubation period at 37°C. Each plate was assayed after their respective incubation periods. P-values generated from two-way analysis of variance with pretreatment and challenge duration as factors affecting viability. * $p < .05$, † $p < .01$, ‡ $p < .001$, § $p < .0001$.

APPENDIX

Figure S1

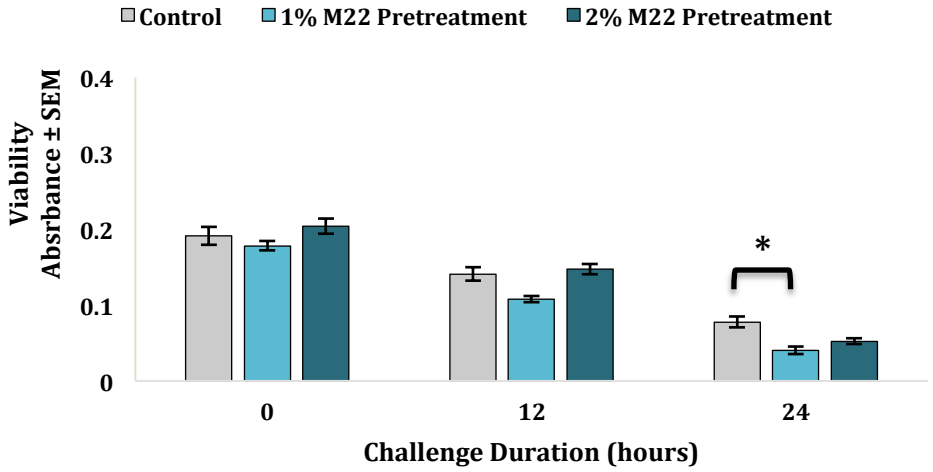

Fig. S1 Absolute viability results from Experiment 1.mESCs had a pretreatment of 0%, 1% or 2% M22 in ESCM for 90 minutes and a recovery period of three hours prior to the challenge. mESC were then seeded at a density of 20,000 cells per well for three identical 96-well plates. The plates were exposed to the challenge of 9% M22 in ESCM for a 0-, 12-, or 24-hour incubation period at 37°C. Each plate was assayed after their respective incubation periods. P-values generated from two-way analysis of variance with pretreatment and challenge duration as factors affecting viability. * $p < .05$, † $p < .01$, ‡ $p < .001$, § $p < .0001$.

Figure S2

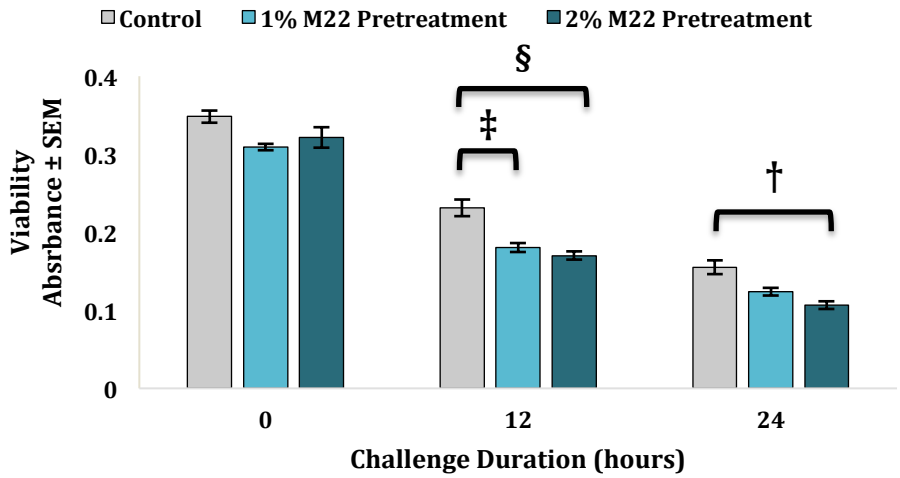

Fig. S2 Absolute viability results from Experiment 2. mESCs had a pretreatment of 0%, 1% or 2% M22 in ESCM for 90 minutes and a recovery period of three hours prior to the challenge. mESC were then seeded at a density of 20,000 cells per well for three identical 96-well plates. The plates were exposed to the challenge of 9% M22 in ESCM for a 0-, 12-, or 24-hour incubation period at 37°C. Each plate was assayed after their respective incubation periods. P-values generated from two-way analysis of variance with pretreatment and challenge duration as factors affecting viability. * $p < .05$, † $p < .01$, ‡ $p < .001$, § $p < .0001$.

Figure S3

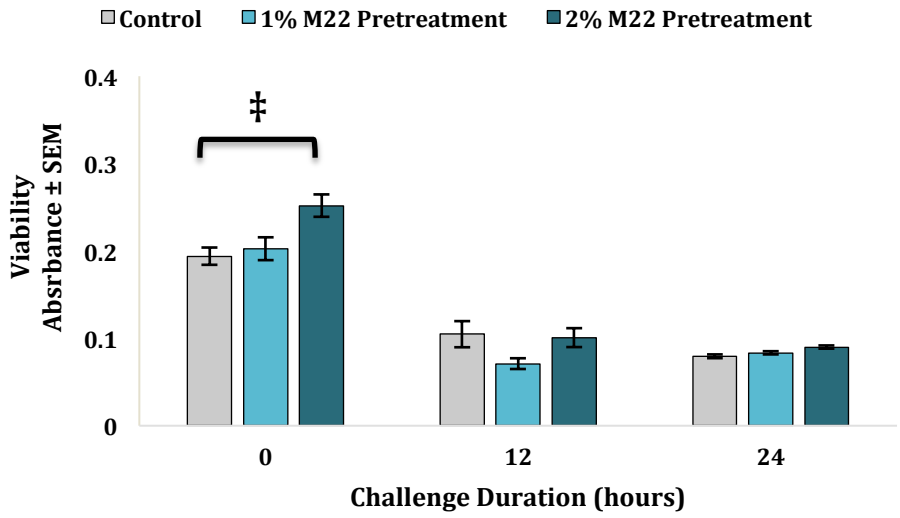

Fig. S3 Absolute viability results from Experiment 3. mESCs had a pretreatment of 0%, 1% or 2% M22 in ESCM for 90 minutes and a recovery period of three hours prior to the challenge. mESC were then seeded at a density of 20,000 cells per well for three identical 96-well plates. The plates were exposed to the challenge of 9% M22 in ESCM for a 0-, 12-, or 24-hour incubation period at 37°C. Each plate was assayed after their respective incubation periods. P-values generated from two-way analysis of variance with pretreatment and challenge duration as factors affecting viability. * $p < .05$, † $p < .01$, ‡ $p < .001$, § $p < .0001$.

Figure S4

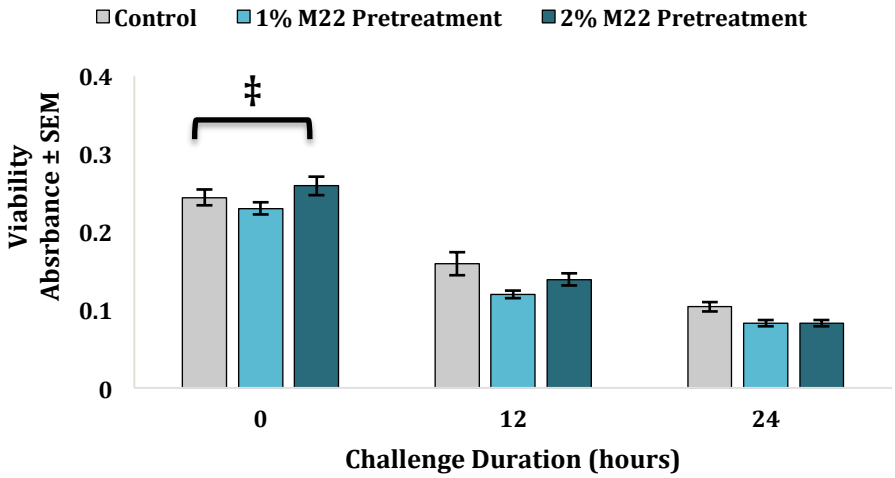

Fig. S4 Summary viability results from absolute data of all experiments. mESCs had a pretreatment of 0%, 1% or 2% M22 in ESCM for 90 minutes and a recovery period of three hours prior to the challenge. mESC were then seeded at a density of 20,000 cells per well for three identical 96-well plates. The plates were exposed to the challenge of 9% M22 in ESCM for a 0-, 12-, or 24-hour incubation period at 37°C. Each plate was assayed after their respective incubation periods. P-values generated from two-way analysis of variance with pretreatment and challenge duration as factors affecting viability. * $p < .05$, † $p < .01$, ‡ $p < .001$, § $p < .0001$.