EFFECTS OF METHYLGLYOXAL ON THE EXTRACELLULAR MATRIX AND ITS INTERACTION WITH CARDIAC CELLS

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Abstract

Cardiovascular disease (CVD) is ranked the second leading cause of death in Canada, with 53,704 heart disease-related deaths documented in 2020 alone. After a patient sustains cardiac injury, such as a myocardial infarction (MI), the heart is often unable to undergo sufficient self-recovery for healthy cardiac regeneration and repair; this is largely attributed to fibrotic tissue development at the injury site and subsequent pathological ventricular remodeling. The prevalence of MI events has created a considerable demand to develop novel strategies for effective and safe post-MI therapies.

Research has indicated that post-MI modifications interfere with endogenous cardiac repair mechanisms, resulting in a pathological state. After an infarction, there is an accumulation of methylglyoxal (MG) at the site of injury. It has been suggested that MG contributes to ventricular fibrotic development, however its underlying mechanism remains unclear. Additionally, the effects that the post-MI cardiac environment, specifically MG accumulation, has on post-MI therapies and biomaterials has not been sufficiently established. Accordingly, the primary focus of this research project is to elucidate the effects of MG on the collagen-rich extracellular matrix (ECM) of the heart and key cardiac cells involved in the repair process. Further, the interaction between MG and a promising collagen-based hydrogel therapy is investigated, exploring the effects of MG on the hydrogel's degradative process. It was found that the MG modification of hydrogels did not alter the degradation rate. Additionally, the degradation products of hydrogels, and MG-modified substrates did not affect the properties and formation of myofibroblasts.

Table of Contents

List of Figures

List of Abbreviations

α-SMA, Alpha-Smooth Muscle Actin; AGE, Advanced Glycation End-product; DAPI, 4′,6-diamidino-2-phenylindole; ECM, Extracellular Matrix; EDC, 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide; FBS, Fetal Bovine Serum; FS, Fisetin; FOV, Field of View; Glo1, Glyoxalase 1; Glo2, Glyoxalase 2; GSH, SH-Glutathione; HBSS, Hank's Balanced Salt Solution; ICC, Immunocytochemistry; ITGA11, Integrin Alpha-11; LDS-PAGE, Lithium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis; MG, Methylglyoxal; MG-H1, Methylglyoxal Derived Hydroimidazolone-1; MI, Myocardial Infarction; MMP, Matrix Metalloproteinase; NHS, N-hydroxysuccinimide; PFA, Paraformaldehyde Solution; PBS, Phosphate Buffer Solution; PBS-T, Phosphate Buffer Solution-Tween 20; ROI, Region of Interest; TCP, Tissue Culture Plate; Tr, Treated; UPLC, Ultra Performance Liquid Chromatography.

Key Words

Biomaterial Degradation; Cardiac Fibroblasts; Cardiac Fibrosis; Collagen-based hydrogel; Collagen Type 1; Extracellular Matrix; Matrix Metalloproteinase-1; Methylglyoxal; Myocardial Infarction; Myofibroblasts; Ventricular Remodeling.

1. Introduction

1.1 Rationale

Cardiovascular disease (CVD) ranks number one in worldwide morbidity (Bayomy et al., 2012). Despite the success of current therapies for myocardial infarction (MI), for ~10% of patients, the ensuing adverse ventricular remodeling can lead to heart failure, which has a 5-year mortality of ~50% (Bayomy et al., 2012). Given that the prevalence of heart failure is growing, there is a need for new strategies to repair the MI heart. Cell-based therapies have demonstrated promise to biologically repair the MI heart; however, post-MI modifications hinder endogenous repair mechanisms. The high mortality rate and prevalence of CVD, specifically MI, motivates further research for the development and optimization of therapeutic strategies for effective cardiac repair. Key concepts and topics for this study are discussed below.

1.2 Myocardial Infarction

A myocardial infarction, or heart attack, occurs when one of the coronary arteries is blocked or has critically low blood flow (*Myocardial Infarction Cardiovascular Research*, 2019). The arterial blockage is often a result of atherosclerotic plaque accumulation, which is the build-up of fatty deposits within the vessels. The section of the heart with no, or limited blood flow caused by the blockage, becomes ischemic and will trigger the infarction (*Myocardial Infarction Cardiovascular Research*, 2019). This can cause significant mechanical stress on the heart which often leads to ventricular remodeling and ultimately heart failure, which can be life-threatening (Humeres & Frangogiannis, 2019).

The treatment plan for recovery and rehabilitation after an MI can be long and strenuous, depending on the severity of the infarction. Current therapies for MI recovery include pharmaceuticals, medical device implants (such as a stent), coronary artery bypass surgery and, in more severe cases, heart transplants. These methods, however, have their drawbacks, including transplant donor shortages, invasive techniques, risk of rejection or infection, thrombosis or stenosis of devices and elongated hospital stays (Miller et al., 2019). Given these disadvantages, the increasing prevalence of heart failure, and the poor cardiac regenerative capacity of a human heart (Li et al., 2021), there is significant demand to develop novel strategies to repair the MI heart.

1.3 Cardiac Fibroblasts

Important players in cardiac repair are the mesenchymal cells called cardiac fibroblasts. The myocardium holds resident fibroblasts enmeshed within the extracellular matrix (ECM) network (Ivey & Tallquist, 2016). The ECM is primarily composed of collagens, proteoglycans, and glycoproteins (Ivey & Tallquist, 2016). The primary role of cardiac fibroblasts is to maintain the structural integrity of connective tissue by manufacturing a variety of ECM proteins and by regulating ECM turnover (Humeres & Frangogiannis, 2019).

In a healthy heart, fibroblasts survey their environment for replacement ECM deposition to maintain cardiac structure and function. After cardiac injury, such as an infarction, cardiac fibroblasts are activated and differentiate into myofibroblasts, exhibiting dynamic phenotypic changes (Camelliti et al., 2005; Oguri et al., 2014). Myofibroblasts display an increase in proliferative and migratory properties due to a higher expression of contractile proteins, such as α-smooth muscle actin (α-SMA) (Oguri et al., 2014). Additionally, the differentiation of fibroblasts

into myofibroblasts increases ECM protein synthesis of the major ECM proteins, collagen type I and type III. The elevated proliferative capacity and collagen production displayed by myofibroblasts plays a major role in the pathological development of fibrotic tissue and scarring, which can cause adverse left ventricular remodeling (Camelliti et al., 2005; Humeres & Frangogiannis, 2019). This left ventricular remodeling reduces cardiac efficiency, decelerates recovery time after an MI, and can ultimately lead to death (Bayomy et al., 2012).

1.4 Methylglyoxal and Methylglyoxal-Derived AGEs

Glycation is a non-enzymatic process by which a carbohydrate covalently bonds to a protein or lipid biomolecule (Lima & Baynes, 2013). Proteins modified through glycation form advanced glycation end-products (AGEs) which are associated with numerous pathologies, including atherosclerosis, diabetes, and neurodegenerative diseases (Blackburn et al., 2017; Lima & Baynes, 2013). By damaging protein structure and function, AGEs can result in tissue dysfunction; this is also known as "dicarbonyl stress" (Chakraborty et al., 2014; Thornalley, 2003). A study by Burr and Stewart exploring the elevated levels of AGEs present in the ECM of diabetic patients indicated a correlation between AGE accumulation and ECM rigidity. The increased rigidity observed was found to be a result of an increase in ECM protein crosslinking, such as collagen (Burr & Stewart, 2020).

AGEs have also been shown to impair post-MI recovery and are linked to heart failure (Thornalley, 2003). Methylglyoxal (MG) is an endogenous dicarbonyl which is highly reactive (Distler & Palmer, 2012). MG accumulates after an MI and is suspected to play a role in cardiac pathologies and adverse remodelling (Distler & Palmer, 2012). When MG reacts with lysine and arginine

protein residues (found abundantly on collagen type I), MG acts as a precursor for AGEs, forming MG-derived AGEs (Blackburn et al., 2017; Lima & Baynes, 2013). As MG is a common precursor for AGEs, it is suspected that MG may contribute to the adverse ventricular remodelling and increased tissue stiffness after an MI (Burr & Stewart, 2020).

After a myocardial infarction, the heart experiences ischemia, inflammation and oxidative stress, all of which trigger MG production and downregulate glyoxalase 1 (Glo1) (Blackburn et al., 2017). Glo1 is an enzyme that participates in methylglyoxal detoxification. Glo1, glyoxalase 2 (Glo2) and the cofactor SH-Glutathione (GSH) are the key components active in the glyoxalase system (Thornalley, 2003). The glyoxalase system works by catalyzing a reaction that converts acyclic α-oxoaldehydes, such as MG, into their corresponding non-toxic α-hydroxyacids, such as d-lactate (Distler & Palmer, 2012). The reduced activity of Glo1 seen after an MI allows for greater MG and MG-derived AGE accumulation.

Studies have indicated that MG accumulation from diabetes is associated with many adverse deleterious effects caused by its abundant intra- and extracellular targets which can lead to cell death, inflammation, and impaired angiogenesis (Blackburn et al., 2017). Less research, however, has been conducted on the MG accumulation seen in non-diabetic related cardiac pathologies.

1.5 Fisetin

Fisetin is a bioactive molecule found in plants, such as strawberries and apples, which is part of the flavonoid group of polyphenols (Khan et al., 2013). It is widely known for its beneficial effects as an antioxidant (Grynkiewicz & Demchuk, 2019). Additionally, it has been shown to promote cytoprotection of normal cells and act as an anti-inflammatory, chemopreventive, and

chemotherapeutic agent (Grynkiewicz & Demchuk, 2019; Khan et al., 2013). With these therapeutic properties, fisetin is often taken as a supplement to reduce the risk of several pathologies including degenerative diseases, cancer, and heart diseases (Pal et al., 2016).

Fisetin has sparked interest in MI recovery therapies due to its antioxidant and anti-inflammatory properties (Eren Cimenci et al., 2022). As a dicarbonyl scavenger, fisetin has been shown to reduce MG-derived AGE accumulation, decrease oxidative stress in the MI heart, which is associated with smaller scar size and improved cardiac function, and increase the level of Glo-1, the major MG-metabolizing enzyme (Eren Cimenci et al., 2022).

1.6 Hydrogel

Hydrogels are hydrophilic polymer networks that are used in a variety of biomedical applications (Ng et al., 2020). Hydrogel therapies have been tested for treating the MI heart. Specifically, our lab has developed an injectable collagen-based hydrogel for this purpose. This treatment involves injecting a hydrogel into the area of cardiac damage to ameliorate and accelerate recovery by acting as a scaffold. A scaffold is a porous biomaterial intended as a temporary support that guides tissue development and cellular organization into a mature and healthy state (Nikolova & Chavali, 2019). It achieves this by supporting and encouraging cell attachment, migration, growth and differentiation and tissue deposition by acting as a three-dimensional support network (Ng et al., 2020; Nikolova & Chavali, 2019). Additionally, hydrogels may be used as a cell- or biomolecule-delivery system to aid in recovery of the infarcted tissue, enhancing its potential as an effective post-MI therapy.

Previous studies have indicated that the collagen-based hydrogel helps to decrease fibrosis, chronic inflammation, and left ventricular thinning (McLaughlin et al., 2019). It has also been shown to improve vascularization and preserve left ventricular ejection fraction (McLaughlin et al., 2019). These observations suggest that the collagen-based hydrogel has a mechanism that allows it to improve the health of the ECM, thus reducing cardiac remodeling.

1.6.1 Components of the hydrogel

In a clinical application, the injectable hydrogel would be delivered through a catheter and deposited at the location of cardiac damage. It would act as a scaffold for new tissue growth and repair (McLaughlin et al., 2019) by mimicking the native cardiac niche to support repair (Geckil et al., 2010). Optimizing the hydrogel for its specific application is key to ensure it meets the biological, mechanical, and physical characteristics needed (Hasan et al., 2015). The hydrogels explored in these studies are composed of water, rat tail collagen type 1, the crosslinkers, 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS), and chondroitin sulfate C (40%).

1.6.1.1 Collagen Type I

Collagen type I is a highly abundant natural polymer found in many tissues, including the heart (Henriksen & Karsdal, 2016). Collagen type I plays a principal role in a tissue's structure and mechanical strength. The primary structure of collagen is a linear chain made up of polypeptides. The secondary structure is the left-handed helical folding of the polypeptide chain. Glycosylation forms the tertiary collagen structure, called the collagen triple helix. Through sulfide-sulfide bond links, three left-handed α chains intertwine into a right-handed superhelix to create the tertiary fibrous structure. Collagen's tertiary structure is what largely contributes to its high

stress-carrying capacity (Fratzl et al., 1998). This will then be further processed to form collagen fibers (Chung et al., 2004). Fibrillar Collagen Type I is formed and secreted into the ECM as a procollagen precursor by fibroblasts (Querejeta et al., 2004). Overexpression of collagen by fibroblasts can lead to pathological fibrotic tissue development, frequently seen in MI patients, resulting in ventricular remodeling and cardiac dysfunction (Querejeta et al., 2004).

Collagen is provided as monomers in an acidic solution (Shoulders & Raines, 2009). When it is in a neutral pH, the collagen monomers will undergo spontaneous fibrilization that is contingent on temperature and concentration (Shoulders & Raines, 2009). Collagen is a top candidate for the basis of hydrogels as it demonstrates high biocompatibility, elicits cellular activity, helps guide tissue repair, and is biodegradable (Hasan et al., 2015). The ability to control gelation at physiological pH and temperature is an additional benefit to using collagen as the basis for the injectable hydrogels.

1.6.1.2 Crosslinkers

Collagen fibres *in vivo* are stabilized through enzymatic reactions leading to crosslinking. For biomaterial synthesis, carbodiimide chemical crosslinking is used to obtain similar physical integrity to mimic that of native tissue (Bax et al., 2017).

EDC and NHS are crosslinkers which preserve the integrity and porous structure of collagen-based hydrogels, and increase resistance against degradation by enzymes (Shepherd et al., 2015). The hydrogel crosslinkers, EDC and NHS, participate in a 2-step coupling reaction between collagen fiber protein residues (Gevorg, 2020). EDC acts as a catalyst to form conjugates between protein molecules through a reaction that activates a carboxyl group which will react with a primary amine

and form an amide bond and an isourea by-product (Gevorg, 2020). NHS is used to enhance coupling efficiency and to increase the stability of the amine-reactive intermediates. Through EDC coupling of NHS to carboxyls, an NHS ester will form, which is substantially more stable than the amine-reactive intermediate (Gevorg, 2020; Shepherd et al., 2015).

A study by Bax et al. demonstrated that EDC/NHS crosslinking uses the same carboxylic side chain chemistry crucial for native-like integrin-mediated cell interactions. Further, crosslinking using EDC and NHS has been shown to result in an aligned collagen fibrillar structure and banding resembling native collagen (Shepherd et al., 2015) and preserves the porous structure of the scaffold, important for cell infiltration and other cellular activities (Yang, 2012). Lastly, EDC and NHS are used because of their thermo-sensitive properties causing gelation at body temperature (37 ℃). This approach allows the hydrogel to be injected as a liquid through a catheter, with gelation initiated upon exposure to body temperature.

1.6.1.3 Chondroitin Sulphate C

Chondroitin sulfate C is biological polymer abundant in the ECM of tissues with important structural and biological functions (Strehin et al., 2010). The addition of chondroitin sulphate C (40%) in the hydrogel works to chemically modify the gel to encourage cell-collagen attachment important for biocompatibility (Hasan et al., 2015).

1.6.2 Scaffold degradation

A key consideration when designing scaffolds is its ability to degrade. Scaffold degradation must occur in tandem with tissue formation while maintaining sufficient structural integrity during

growth and remodeling (O'Brien, 2011). The environmental conditions including pH, the presence of enzymes, and cells found in that tissue can influence degradation of a biomaterial (O'Brien, 2011).

With elevated levels of MG present post-MI, it is important to consider its effects on the degradation rate of the hydrogel, as well as any other alterations it may have on the hydrogel as a potent glycating agent. The length of time the hydrogel is present in the heart, and the degradation products of the hydrogel, may impact treatment efficacy and alter ECM and cellular interactions.

1.7 Objectives and Hypotheses

Herein, this study sought to investigate interactions between MG, key cardiac cells participating in MI-recovery, and a collagen-based hydrogel therapy. The primary objective was to evaluate the degradation process of an MG-modified hydrogel and investigate its interaction with cardiac fibroblasts.

It was hypothesized that the collagen hydrogels would exhibit resistance to degradation due to the MG-modifications conferred on the collagen matrix structure. Additionally, it was hypothesized that MG-modified collagen and the degradation products of MG-modified hydrogels would prompt the differentiation of fibroblasts to their activated myofibroblast form seen during fibrotic tissue development.

2. Methods

2.1 Hydrogel preparation

A collagen-based hydrogel was prepared using a T-piece system. The T-piece system was primed with phosphate buffered saline (PBS) to ensure no bubbles were trapped in the T-piece or syringes. PBS was carefully removed and 1 mL of rat tail collagen ((3.38 mg/mL), sc-136157, Santa Cruz Biotech, Dallas, TX, USA) was added into the system. Next, 100 uL of 40% chondroitin sulfate was added followed by 0.015 grams of EDC (E7750-100MG, Sigma Aldrich, St. Louis, MO, USA) and 0.025 g of NHS (157270250**,** Thermo Fisher, Waltham, MA, USA). The solution was mixed within the T-piece system and kept on ice. The hydrogels were brought to a neutral pH by the addition of sodium hydroxide. The gel was then spun in an Eppendorf MiniSpin 5452 Microcentrifuge **(**Z666491, Millipore Sigma, Burlington, MA, USA) at 80,000 RPM for 60 seconds. The gel was incubated at 37 °C for 45 minutes after being aliquoted into the appropriate plate for the assay.

2.2 Cardiac Fibroblast Isolation

Donor hearts were acquired from the Civic Hospital Operating Room. The donor hearts were from patients who died from reasons unrelated to CVD (example: neurologic determination or circulatory determination of death (NDD; DCD) organ donors). Patient information and their specific cause of death could not be disclosed for patient confidentiality purposes. The hearts were transported to the University of Ottawa Heart Institute for storage and cardiac fibroblast isolation. The Ottawa Health Science Network Research Ethics Board granted approval for the collection of and experimentation on human cardiac tissue and human cardiac fibroblasts.

For cell isolation, a 3x3 centimeter piece of myocardium was cut above the apex of the left ventricle. Any fat surrounding the piece of the myocardium was discarded. Using a scalpel and blade, the piece was cut into very small pieces. A digestion buffer was prepared using 0.5 grams of Dispase II (0.8 U/mg) **(**17105041, Thermo Fisher, Waltham, MA, USA), 0.66 grams of Collagenase B **(**11088807001, Millipore Sigma, Burlington, MA, USA) from Clostridium Histolyticum and 1.5 mL of 5 M Sodium chloride. An extraction buffer was then prepared using 4666 uL of Hank's balanced salt solution (HBSS) (H9269, Millipore Sigma, Burlington, MA, USA) and 2000 uL of the prepared digestion buffer. HBSS is used to maintain pH and osmolality of the cells to enable the cell viability outside of the growth medium (Holmbeck & Birkedal-Hansen, 2013). The extraction buffer was added to the heart tissue sample and incubated at 37 °C for 30 minutes, vortexing the mixture every 5 minutes. Next, 38.334 mL of HBSS was added to the mixture to neutralize the digestion buffer. It was then centrifuged for 5 minutes at 600 g in an Eppendorf Centrifuge 5804 (EP022628146, Millipore Sigma, Burlington, MA, USA). The supernatant was removed, and the remaining pellet was dissolved in 45 mL of HBSS.

The sample was then filtered through a 100 um strainer (22-363-549, FisherScientific, Waltham, MA, USA) a 70 um strainer (22-363-548, FisherScientific, Waltham, MA, USA) and a 40 um strainer (08-771-1, FisherScientific, Waltham, MA, USA). The sample was centrifuged for 5 minutes at 350 g in an Eppendorf Centrifuge 5804 three times, or until the supernatant was clear. The pellet was resuspended in 15 mL of cardiac fibroblast growth medium, composed of Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 (DMEM/F-12, HEPES (11330032, Thermo Fisher, Watham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (12483020, Thermo Fisher, Watham, MA, USA), 1% Penicillin-Streptomycin **(**15140122, Thermo

Fisher, Watham, MA, USA) and 1:1000 gentamicin (15750-060, Thermo Fisher, Watham, MA, USA). The cell suspension was seeded in a T75 cell culture flask (156499**,** Thermo Fisher, Watham, MA, USA) and incubated at 37 °C overnight. The cardiac fibroblast growth medium was changed the next day and every 2-3 days after that. The cells were visualized under a microscope 1-2 days after seeding. At approximately 85-90% confluency, the cells can be frozen in a cryotank and stored for later use.

2.3 Hydrogel Mass Spectrometry

A hydrogel was prepared and incubated at 37 °C overnight, covered in 5 mL of PBS, 25 uM MG (M0252, Sigma Aldrich, St. Louis, MO, USA) diluted in PBS, 100 uM MG diluted in PBS or 1 mM MG diluted in PBS. The next day, 90 uL of each sample was aliquoted into separate 0.5 mL Eppendorf tubes.

The samples then underwent a reduction step by adding 200 uL of sodium borohydride (100 mmol/L) dissolved in a borate buffer (pH 9.2/200 mmol/L). The samples were incubated for 2 hours at room temperature. Next, 1000 uL of trifluoroacetic acid was added as a deproteinization step. The sample were centrifuged at 4300 x g for 20 minutes in 4 °C using a Sorvall Legend Micro 17 centrifuge (75002441, Thermo Fisher, Watham, MA, USA). The supernatant was carefully aspirated and then 500 uL of 6N hydrochloric acid was added to each pellet. They were then incubated at 110°C for 18 hours, as a hydrolysis step.

Next, 40 uL of hydrolysate and 20 uL of internal standard were mixed in a reaction vial and then evaporated to dryness under a stream of nitrogen gas at 70°C. After the first evaporation, 100 uL

of Butanol-Hcl (3:1) was added as a derivatization step. It was then incubated for 90 minutes at 70°C and then evaporated to dryness under a stream of nitrogen gas. Lastly, 100 uL of double distilled water was added to the samples.

The samples were analyzed by Ultra Performance Liquid Chromatography (UPLC) to detect the concentration of methylglyoxal-derived hydroimidazolone-1 (MG-H1), an MG-derived AGE that is formed when MG reacts with the arginine residue (Wetzels et al., 2019).

2.4 Gel Electrophoresis

A lithium dodecyl sulfate polyacrylamide gel electrophoresis (LDS-PAGE) was conducted to analyze the degradation products of MG-modified and unmodified hydrogels. Three hydrogels were prepared and 200 uL were deposited on three 6-well plates (07-200-83, Thermo Fisher, Watham, MA, USA). After gelation, 5 mL of 1 mM MG diluted in PBS or 5 mL of PBS, was added to cover the hydrogels. The hydrogels were then incubated at 37°C overnight. After 24 hours, the solutions were aspirated, and the gels were thoroughly washed in PBS. Type I Collagenase (9001-12-1) was diluted to 5 U/mL using 0.1 M Tris Base, adjusted to pH 7.4 using hydrochloric acid and complemented with 5 mM calcium chloride. The Type I Collagenase Solution was then added to cover and degrade each hydrogel. After 6 hours, the solution containing 5 U/mL Collagenase I and the degraded hydrogel was placed in a test tube and spun down for 20 minutes in 4 ℃ at 15,000 RPM using a Sorvall RC-6 Plus Centrifuge (46910, Thermo Fisher, Watham, MA, USA). The supernatant was carefully removed and 50 uL of each sample was aliquoted into 0.5 mL Eppendorf tubes.

Next, 12.5 uL of Pierce LDS Sample Buffer, Non-Reducing (4X) (84788, Thermo Fisher, Watham, MA, USA) was added to 37.5 uL of the protein samples and incubated at 95°C for 5 minutes. Then, 45 uL of the solution samples were loaded into a 4 to 12%, Tris-Glycine, 1.0 mm, Mini Protein Gel (XV04120PK20, Thermo Fisher, Watham, MA, USA). A Protein Molecular Weight Standard (broad range) (P6649, Thermo Fisher, Watham, MA, USA) was used as a ladder. The gels were run at 120V for 15 minutes and then 185V for 35 minutes using a Mini Gel Tank (A25977, Thermo Fisher, Watham, MA, USA).

The hydrogels were removed and washed thoroughly in ultra-pure water. They were then stained using GelCode™ Blue Safe Protein Stain (24594, Thermo Fisher, Watham, MA, USA) for 4 hours, and destained overnight in ultrapure water.

2.5 Collagen-based hydrogel degradation

Assays were conducted to assess the degradation rate of MG-modified hydrogels versus unmodified hydrogels. For the following three degradation assays, hydrogels deposited on a D × H 150 mm × 25 mm pre-weighed Petri dish (CLS430599-5EA, Millipore Sigma, Burlington, MA, USA) were covered in a degradation solution (varied based on the assay) and weighed at various time points throughout the degradation process. The percent residual mass was calculated using Equation (1):

$$\% \text{ Residual Mass} = [1-((m_0-m_t)/m_0)] \times 100, \quad (1)$$

Where m_0 is the mass at time(t)=0, and m_t is the mass at time(t)=x in the degradation process.

2.5.1 Hydrogel degradation using Collagenase I Solution

Hydrogels were prepared and 180 uL of the hydrogel was deposited on the pre-weighed Petri-dish. 5 mL of 100 uM of MG diluted in PBS was added to three of the wells and 5 mL of PBS was added to the other three wells. After 24 hours, the solutions covering the hydrogels were aspirated and the hydrogels were thoroughly washed in PBS. Next, 5 mL of 5 U/mL Collagenase I Solution was added to cover the hydrogels. Every hour, the Collagenase solution was removed, and the hydrogels were weighed. This was repeated until the hydrogels were completely degraded. This experiment was repeated using PBS rather than the Collagenase I Solution, as a control.

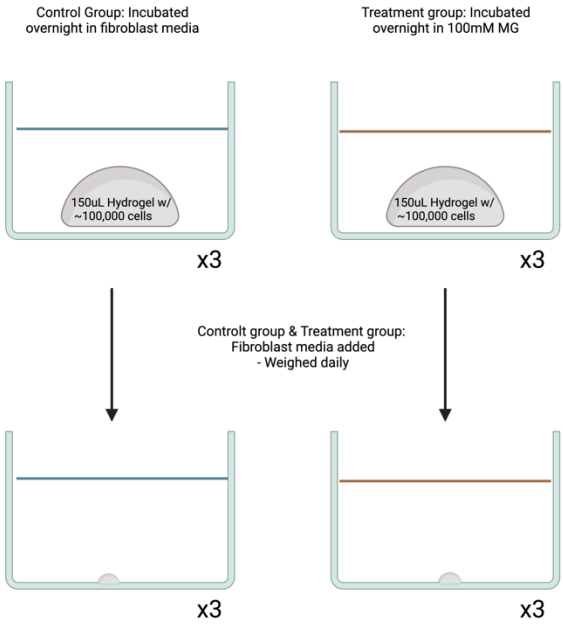

Figure 1 Schematic of the procedure used for hydrogel degradation using Collagenase I Solution and Cardiac fibroblasts when exposed to MG overnight

2.5.2 Cardiac Fibroblast Degradation - Hydrogels treated in MG overnight

Approximately 100,000 human cardiac fibroblast cells were directly mixed into a hydrogel. This was done in sterile conditions. Three of the prepared cardiac fibroblast-loaded hydrogels deposited on pre-weighed Petri-dishes were covered in 5 mL of 100 uM MG diluted in cardiac fibroblast growth medium, and three were covered in 5 mL of cardiac fibroblast growth medium, alone.

These were incubated at 37 ℃ overnight. The next day, the medium was aspirated, and the hydrogels were weighed (t=0). 5 mL of cardiac fibroblast growth medium was added to cover all the hydrogels and stored in 37 ℃. Daily, the cardiac fibroblast growth media was removed, and the hydrogels were weighed until fully degraded. Each time the media was removed, it was replaced with cardiac fibroblast growth medium, alone.

2.5.3 Cardiac Fibroblast Degradation - Hydrogels treated in 25 uM MG everyday

Similar to the former protocol, a hydrogel was prepared with approximately 100,000 human cardiac fibroblast cells directly mixed into the hydrogel, done in sterile conditions. Then, 150 uL of the prepared cardiac fibroblasts-loaded hydrogels were deposited into 6 pre-weighed wells and incubated overnight at 37 ℃ in cardiac fibroblast growth medium, without methylglyoxal. The next day, the medium was aspirated, and the hydrogels were weighed (T=0). Three of the prepared hydrogels were then covered in 5 mL of 25 uM MG diluted in cardiac fibroblast growth medium, and three covered in 5 mL of cardiac fibroblast growth medium, alone. The media was aspirated daily, and the hydrogels were weighed until the hydrogels completely degraded. Each time the media was removed, the media in three of the wells was replaced with 25 uM MG diluted in cardiac fibroblast growth medium, and the other three wells were covered in cardiac fibroblast growth medium only.

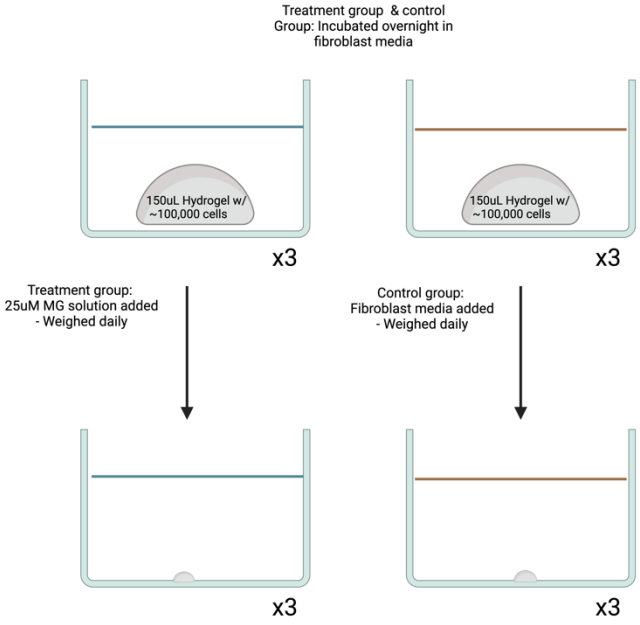

Figure 2 Schematic of the procedure used for degradation using Cardiac fibroblasts when exposed to MG daily

2.6 Immunocytochemistry

For the following three immunocytochemistry (ICC) procedures, the following general ICC protocol was used:

Cardiac fibroblasts were cultured under appropriate conditions for the various assays. Media from the wells was aspirated and then wells were washed with PBS. The cells were fixed in place by adding 300 uL of 4% paraformaldehyde solution (PFA at 4%) and left for 10 minutes on a shaker. PFA (4%) was aspirated, and PBS was used to wash the wells. A 10% FBS solution, diluted with PBS, was added to the wells, and left for 1 hour to act as a blocking agent. The primary antibody of interest was diluted in 10% FBS to the appropriate concentration and added to the wells. They were left overnight on a shaker in 4 ℃. The next day, the primary antibody was aspirated, and wells were washed thoroughly with PBS treated with Tween 20 (28320**,** Thermo Fisher, Watham, MA, USA) (PBS-T). The secondary antibodies were diluted to the appropriate concentration and added to the wells, keeping the assays in the dark. Fluorescence microscopy was used for imaging. Processing and analyzing was completed using Zen3.4 and ImageJ software.

For the following ICC trials, three values were measured. The percentage of α-SMA was considered for assays which incubated cells in their respective conditions for 48 hours. For assays which used a 72-hour incubation period, it was observed that most cells were expressing either integrin α-11 (ITGA11) or α-SMA, therefore, the mean intensity and integrated densities were also considered. The mean intensity measures the average pixel intensity values of the region of interest (ROI). Integrated density is the total sum of pixel values in the ROI (*Analyze Menu*) These values were used to measure protein expression to compare between conditions.

2.6.1 Cardiac fibroblasts treated with degraded hydrogel products +/- methylglyoxal

Hydrogels were prepared and 150 uL was deposited into 9 wells on a 6-well TCP (tissue culture plate). After gelation, the hydrogels were covered with either 5 mL of 1 mM MG diluted in PBS, or in 5 mL of PBS alone. The hydrogels were incubated at 37°C overnight. After 24 hours, the solutions were aspirated, and the gels were thoroughly washed in PBS. To degrade the hydrogel, 5 mL of 5 Units/mL Collagenase I solution was added to the wells to fully cover each gel. After 6 hours, the solution containing 5 Units/mL Collagenase I and the degraded hydrogel was transferred to a test tube and spun for 20 minutes in 4 ℃ at 15,000 RPM using a Sorvall RC-6 Plus Centrifuge (46910, Thermo Fisher, Watham, MA, USA). The supernatant was carefully removed, and the pellet was diluted with cardiac fibroblast medium. Approximately 20,000 cardiac fibroblasts were seeded on a 24-well TCP (32190102, Millipore Sigma, Burlington, MA, USA) in cardiac fibroblast growth medium +/- the products of the degraded hydrogel. Cells were also cultured in cardiac fibroblast growth medium alone, to act as a control. Technical triplicates of each condition were conducted. The assays were incubated in 37 ℃ for 72 hours.

For this assay, ITGA11 and α-SMA were the proteins of interest. The primary antibody used to investigate the ITGA11 expression was the Anti-ITGA11 antibody (ab198826, Abcam, Cambridge, UK) at a 1/200 dilution factor and the primary antibody Anti-alpha smooth muscle Actin antibody (ab5694, Abcam, Cambridge, UK) used to investigate the α-SMA expression was at a 1/250 dilution factor.

The secondary antibody used was Alexa Fluor 594 donkey Anti-Rabbit (A32733, Abcam, Cambridge, UK) at a 1/600 dilution factor. This secondary antibody was compatible for both

primary antibodies. 4′,6-diamidino-2-phenylindole (DAPI) (10236276001, Millipore Sigma, Burlington, MA, USA) was added to the wells at a 1/100 dilution factor.

2.6.2 Collagen plate with cardiac fibroblasts treated +/- methylglyoxal and +/- fisetin

A 24-well Collagen Type I plate (A1142802, Thermo Fisher, Waltham, MA, USA) was treated overnight at 37°C, with 500 uL of PBS in 12 wells and 500 uL of 1 mM methylglyoxal in the remaining 12 wells. The next day, the wells were thoroughly washed with PBS. Cardiac fibroblasts were distributed equally in either cardiac fibroblast growth medium alone, in fisetin (F4043, Sigma Aldrich, St. Louis, MO, USA) diluted to 10 uM in cardiac fibroblast growth medium, in 25 uM MG diluted in cardiac fibroblast growth medium or in 10 uM fisetin and 25 uM methylglyoxal diluted in cardiac fibroblast growth medium. Approximately 20,000 cardiac fibroblasts were seeded into each well, with technical triplicates of each condition. The assays were then incubated in 37 ℃ for 72 hours.

ITGA11 was the protein of interest for this assay. The primary antibody used to investigate the ITGA11 expression was the Anti-ITGA11 antibody. The optimal concentration to use for the ICC application was determined to be 1/200. The secondary antibody used was Alexa Fluor 594 donkey Anti-Rabbit at a 1/600 dilution factor. DAPI was used to better identify the cells at a 1/1000 dilution factor.

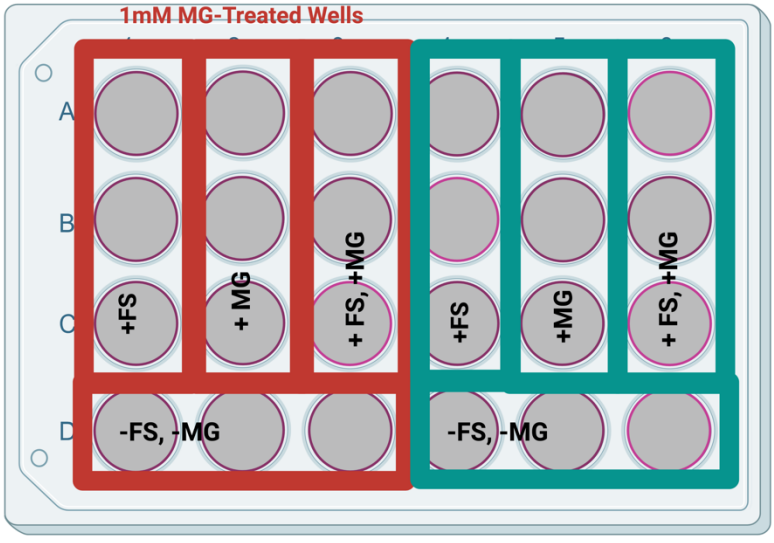

Figure 3 Schematic of experimental setup for cardiac fibroblast cultures on a collagen-coated plate under different treatment conditions.

2.6.3 Cardiac fibroblasts treated with +/- methylglyoxal

A 24-well Collagen Type I plate was treated overnight at 37 ℃, with 5 mL of PBS in 6 wells and 5 mL of 1 mM MG in 6 wells. A 24-well TCP was also treated overnight at 37°C, with 5 mL of PBS in 6 wells and 5 mL of 200 uL human plasma fibronectin (FC010**,** Sigma Aldrich, St. Louis, MO, USA) diluted in 30 mL PBS in 6 wells. The next day, the wells were thoroughly washed with PBS. Approximately 20,000 cardiac fibroblasts in cardiac fibroblast growth medium were seeded in each well. Technical triplicates of each condition were conducted. The assays were incubated in 37°C for 48 hours.

α-SMA was the protein of interest for this assay. The primary antibody used to investigate the α-SMA expression was anti-alpha smooth muscle actin antibody at a 1/250 dilution factor. The secondary antibody used was Alexa Fluor 594 donkey Anti-Rabbit at a 1/600 dilution factor. DAPI was added to the wells at a 1/100 dilution factor.

2.7 Adhesion Assay

A 24-well Collagen Type I plate was treated overnight at 37°C, with 5 mL of PBS in 6 wells and 5 mL of 1 mM MG in 6 wells. A 24-well TCP was also treated overnight at 37 ℃, with 5 mL of PBS in 6 wells and 5 mL of fibronectin dilution in 6 wells. The next day, the wells were thoroughly washed with PBS. Approximately 20,000 cardiac fibroblasts in cardiac fibroblast growth medium were seeded in the treated wells. Technical triplicates of each condition were conducted. The assays were incubated in 37 ℃ for 1 hour. Next, the media was aspirated and DAPI, at a 1/1000 dilution factor diluted in PBS, was added to each well, keeping plates in the dark. Images were taken using an inverted microscope and the cell count per field of view was recorded.

3. Results

3.1 Hydrogel Mass Spectrometry

A mass spectrometry analysis was conducted to test the hypothesis that hydrogels exposed to higher concentrations of MG would have higher MG-H1 levels, indicating a modified hydrogel through MG-collagen glycation.

No MG-H1 was detected in the hydrogels incubated overnight in PBS alone (Fig. 4). Hydrogels treated overnight with 25 uM, 100 uM and 1 mM of methylglyoxal expressed MG-H1 and showed a positive correlation between methylglyoxal concentration with the amount of MG-H1 that was formed (Fig. 4).

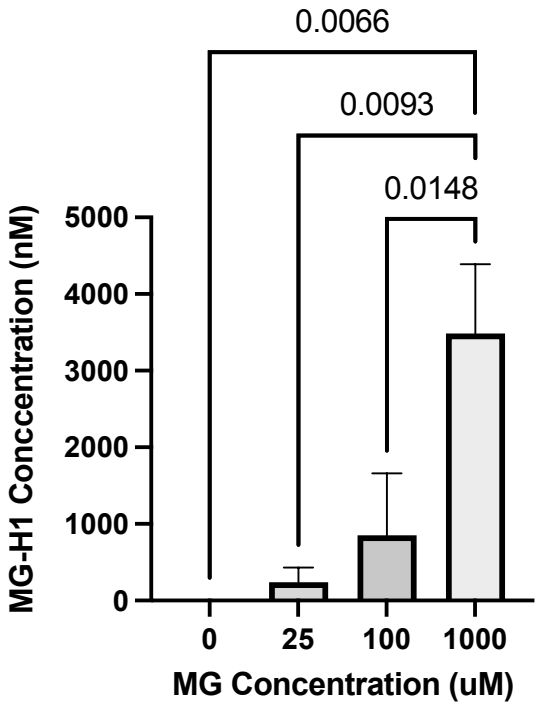

Figure 4 MG-H1 formation for hydrogels incubated in different MG concentrations. P-values of the pairwise comparisons are indicated by the value above each bracket (n=3).

A One-Way ANOVA indicated statistical significance between conditions (p=0.0042). Tukey's multiple comparisons test indicated statistical significance between 1000 uM vs 0 uM (p=0.0066), 1000 uM vs 100 uM (p=0.0093) and 1000 uM vs 25 uM (p=0.0148).

3.2 Degradation Assays

3.2.1 Hydrogel Degradation

Three hydrogel degradation rate assays were run to test the hypothesis that a MG-modified hydrogel would increase the rate of degradation of the hydrogel.

3.2.1.1 Hydrogel degradation using Collagenase I Solution

Using a Collagenase I solution, hydrogels were degraded and the weight throughout the degradation process was recorded (Fig. 5). As anticipated, there was a negative correlation between % residual mass and time, showing that % residual mass of the hydrogel decreased as time passed. The hydrogel was completely degraded after 5 hours of exposure to Collagenase I solution.

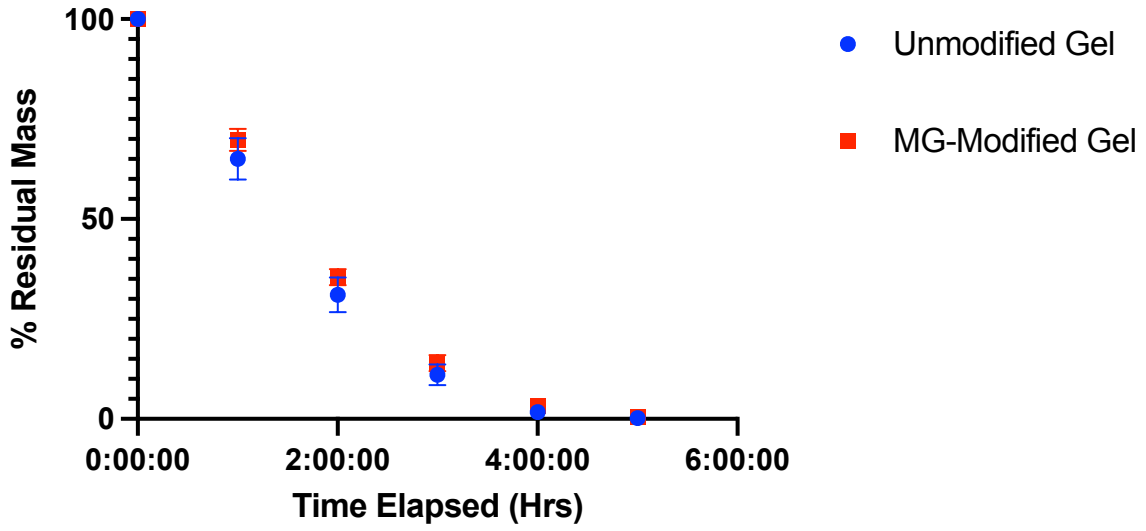

Figure 5 100 uM MG-modified and unmodified hydrogel degraded in a Collagenase I solution (n=3).

An unpaired T-test was completed, indicating that there is no statistical significance between unmodified and MG-modified hydrogels at any time-point (df=10, t(df) = 0.1014, p = 0.9213).

3.2.1.2 Cardiac Fibroblast Degradation - Hydrogels treated in 100 uM MG overnight

Using cardiac fibroblasts that were directly mixed into the hydrogels during preparation, hydrogels were degraded. The weight throughout the degradation process was recorded. The MG-modified hydrogels were treated with 100 uM of MG diluted in cardiac fibroblast growth media overnight. Again, the % residual mass of the hydrogel decreased as time increased (Fig. 6). The hydrogel was degraded after 12 days of cardiac fibroblast exposure.

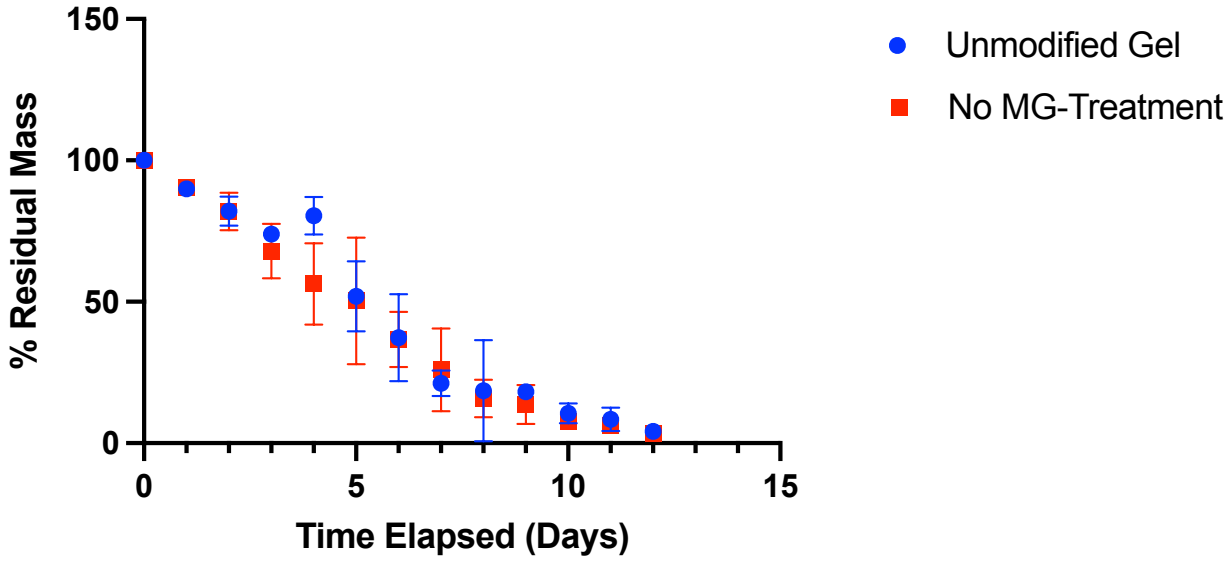

Figure 6 100 uM MG-modified and unmodified hydrogel degraded by cardiac fibroblasts (n=3).

An unpaired T-test was used, indicating that there is no statistical significance between unmodified and MG-modified hydrogels at any time-point (df=24, t(df)=0.2374, p=0.8143).

3.2.1.3 Cardiac Fibroblast Degradation - Hydrogels treated in 25 uM MG everyday

Again, after adding cardiac fibroblasts directly into the hydrogels during preparation, hydrogels were degraded. The weight throughout the degradation process was recorded. Daily, 25 uM of MG diluted in new cardiac fibroblast media was added to the MG-modified hydrogels. Consistent with the last two degradation assays, the % residual mass of the hydrogel decreased overtime (Fig. 7). The hydrogel was degraded after 8 days of cardiac fibroblast exposure.

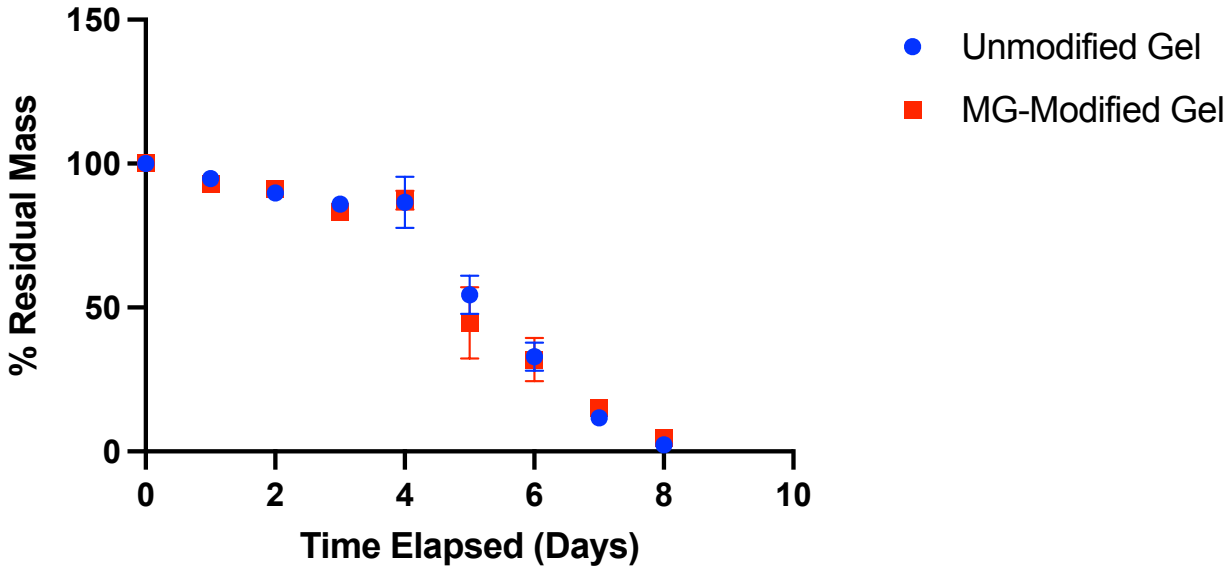

Figure 7 Unmodified hydrogels and hydrogels exposed to 25 uM methylglyoxal daily, degraded by cardiac fibroblasts (n=3).

An unpaired T-test was used to analyze data, indicating that there is no statistical significance between unmodified and MG-modified hydrogels at any time-point (df=16, t(df)=0.04902, p=0.9615).

3.2.2 LDS-PAGE

An LDS-PAGE was conducted to detect differences between MG-modified and unmodified collagen degradation products after MMP-1 degradation. A single band was detected near the inlet of each well, at approximately 300,00 Da, indicating that a separation of degradation products was not achieved under the test conditions used. Figure 8 demonstrates unmodified hydrogel degradation products with and without additional heat denaturation prior to the LDS-treatment. The far-left column shows the molecular weight standard.

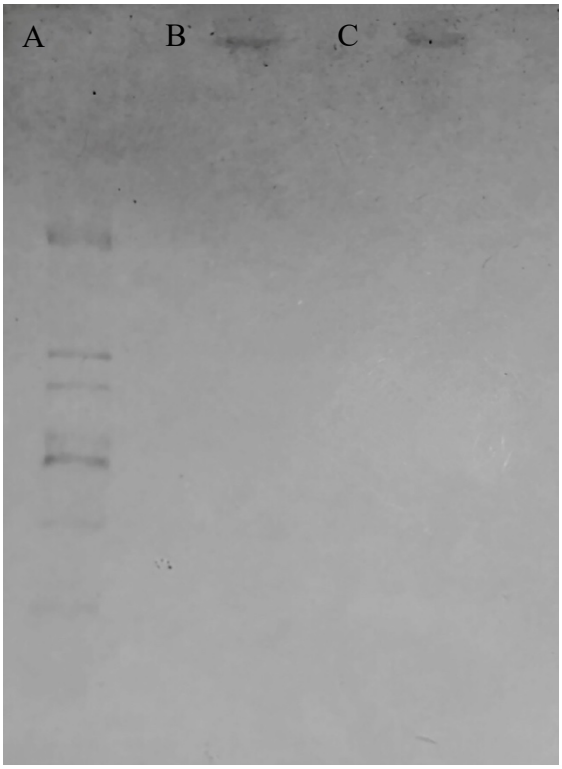

Figure 8 ***Image of an LDS-PAGE gel loaded with a molecular weight standard (A), degraded hydrogel product (B), denatured degraded hydrogel product (C),***

3.2.3 Cardiac Fibroblasts Expression of ITGA11 after exposure to modified or unmodified hydrogel degradation product

An ICC assay was conducted to test the hypothesis that exposure of cardiac fibroblasts to MG-modified hydrogel degradation products will elicit higher expression of ITGA11 compared to those exposed to unmodified hydrogel degradation products or cardiac fibroblast growth medium alone. Representative images of each condition are shown in Fig. 9. The three measurements considered are the % of ITGA11 expressed, the mean intensity per cell and the integrated density per cell. Cardiac fibroblasts from three heart donors were used for these assays (n=3).

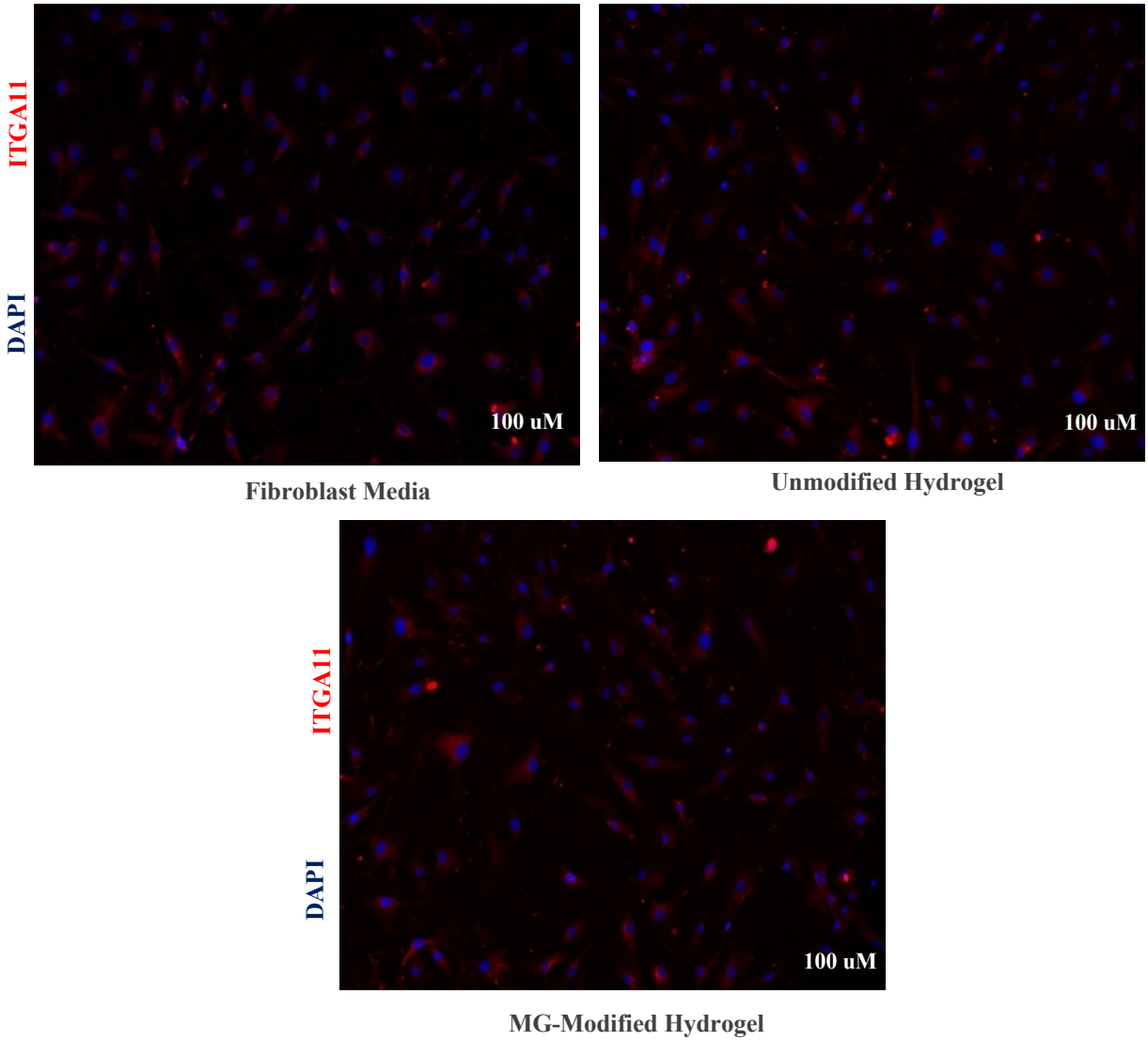

Figure 9 Representative images of cellular expression of ITGA11 for cardiac fibroblasts cultured in fibroblast medium or diluted degraded hydrogel product +/-MG treatment. Blue = DAPI, red = ITGA11.

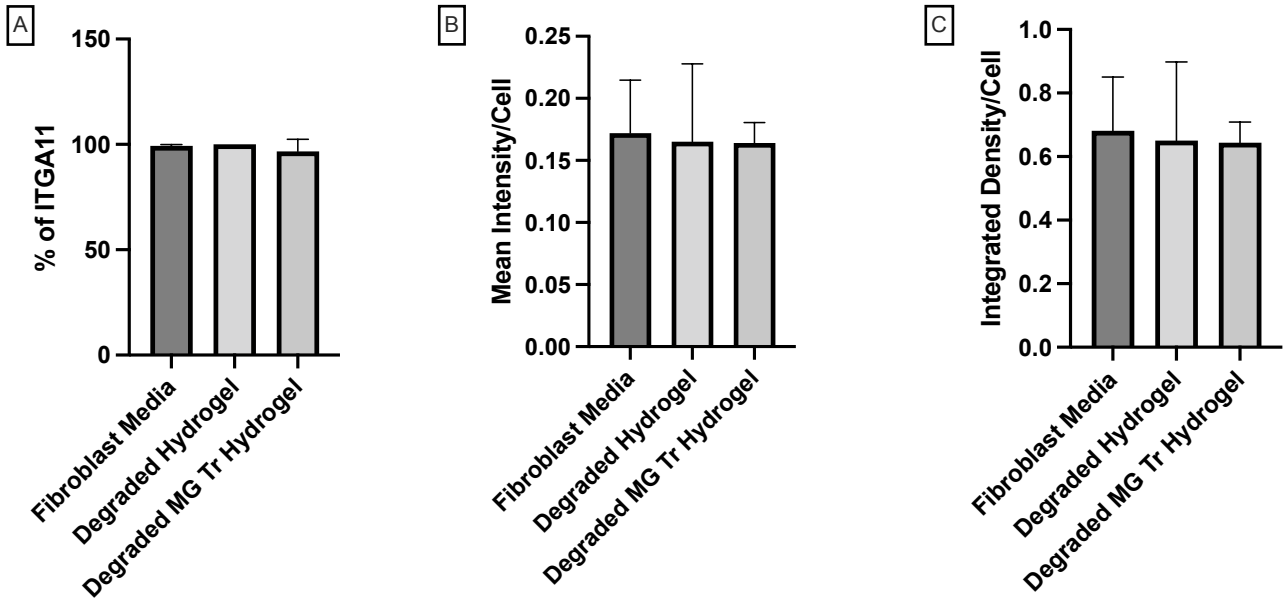

Figure 10 The percentage of cells expressing ITGA11(A), the mean intensity of ITGA11 expression per cell (B) and the integrated density of ITGA11 expression per cell (C) when cultured in cardiac fibroblast medium or diluted degraded hydrogel product +/-MG treatment (n=3).

A One-Way ANOVA indicated no statistical significance between the % of ITGA11 expressed (Fig. 10; p=0.5929). No significant differences were found between mean intensity per cell (Fig. 10; p=0.9723) or integrated density per cell (Fig. 10; p=0.9635). Tukey's multiple comparisons test indicated no statistical significance between pairs of each condition.

3.2.4 Cardiac Fibroblast Expression of α-SMA after exposure to modified or unmodified hydrogel degradation products

To assess cardiac fibroblast expression of α-SMA under various conditions, ICC was conducted. This assay was run to test the hypothesis that cardiac fibroblasts treated with MG-modified hydrogel degradation product will elicit higher expression of α-SMA compared to cardiac

fibroblasts treated with unmodified hydrogel degradation product or treated with cardiac fibroblast growth medium alone. Representative images of each condition are shown in Fig. 11. The three measurements considered are the % of cells expressing α-SMA, the mean intensity per cell and the integrated density per cell. Cardiac fibroblasts from three heart donors were used to for these assays (n=3).

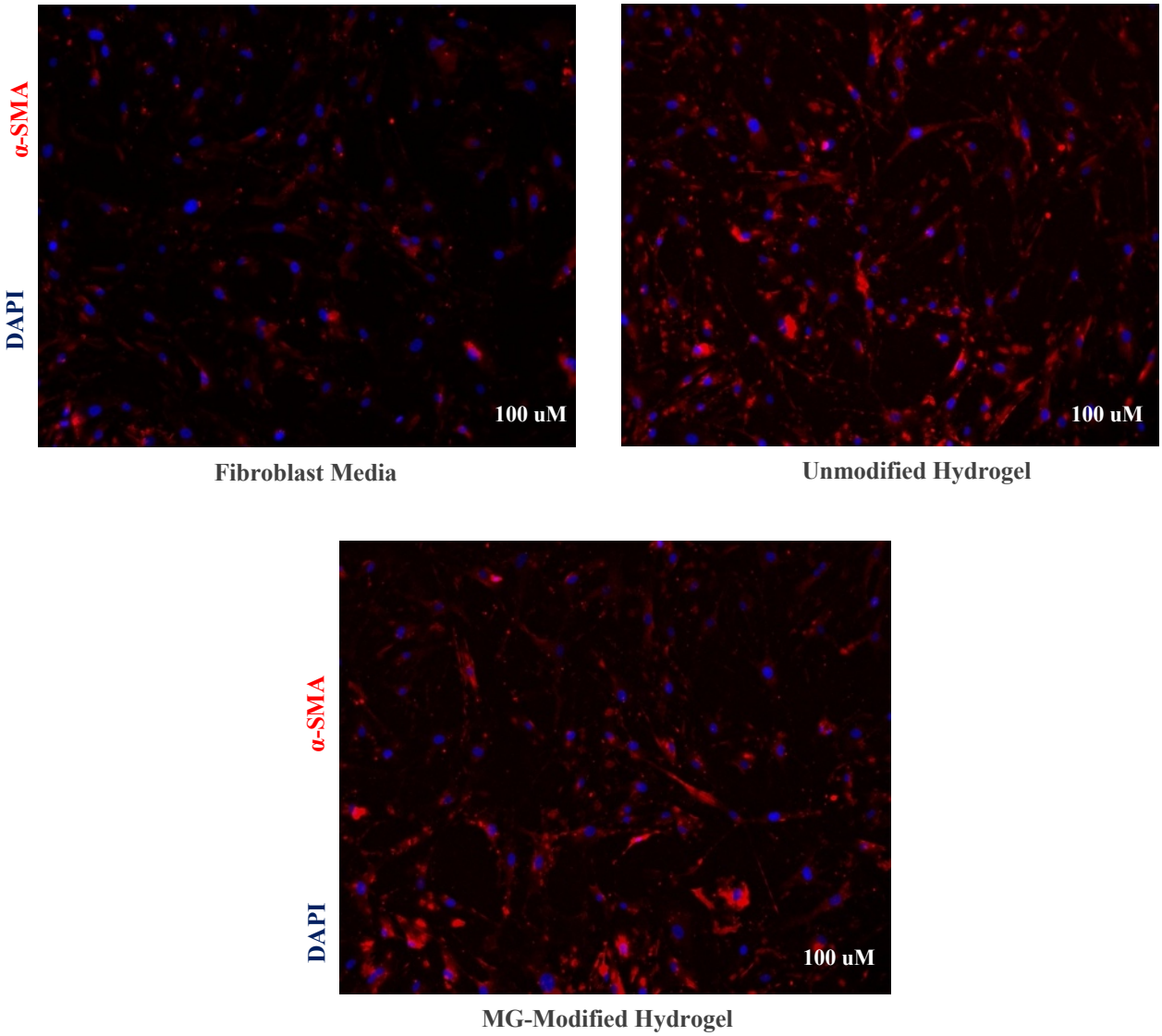

Figure 11 Representative images of cellular expression of α-SMA when cultured in cardiac fibroblast medium or diluted degraded hydrogel product +/-MG treatment. Blue = DAPI, red = α-SMA.

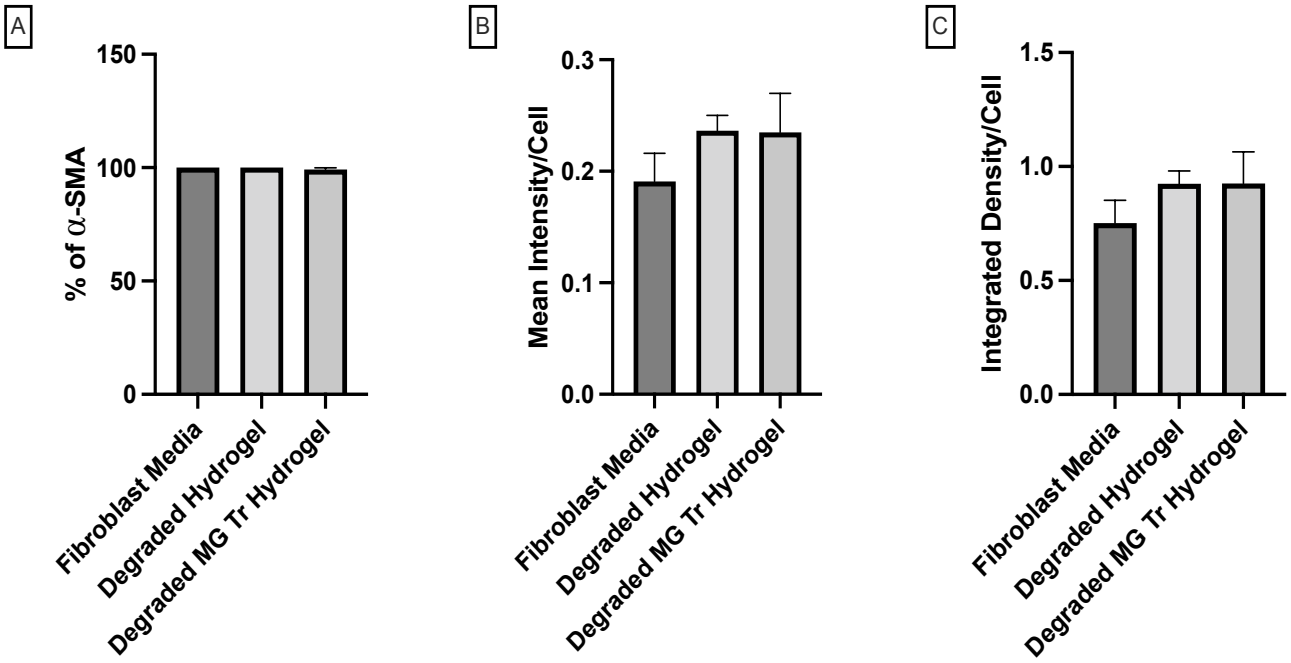

Figure 12 The percentage of cells expressing α-SMA (A), the mean intensity of α-SMA expression per cell (B) and the integrated density of α-SMA expression per cell (C) when cultured in cardiac fibroblast medium or diluted degraded hydrogel product +/-MG treatment (n=3).

A One-Way ANOVA indicated no statistical significance between of the % of α-SMA (Fig. 12A; p=0.8577). No significant differences were found between mean intensity per cell (Fig. 12B; p=0.1308) or integrated density per cell (Fig. 12C; p=0.1383). Tukey's multiple comparisons test indicated no statistical significance between pairs of each condition.

3.3 ICC and Adhesion Assays on MG-Modified and Unmodified Collagen

3.3.1 Cardiac Fibroblast α-SMA expression on Modified VS Unmodified Type I Collagen Plate

An ICC assay was conducted to test the hypothesis that cardiac fibroblasts cultured on an MG-modified collagen plate will express an elevated level of α-SMA compared to cardiac fibroblasts cultured on unmodified collagen plates. The measurement determined for each condition was the % of α-SMA expressed. Cardiac fibroblasts from five heart donors were used to for this assay (n=5).

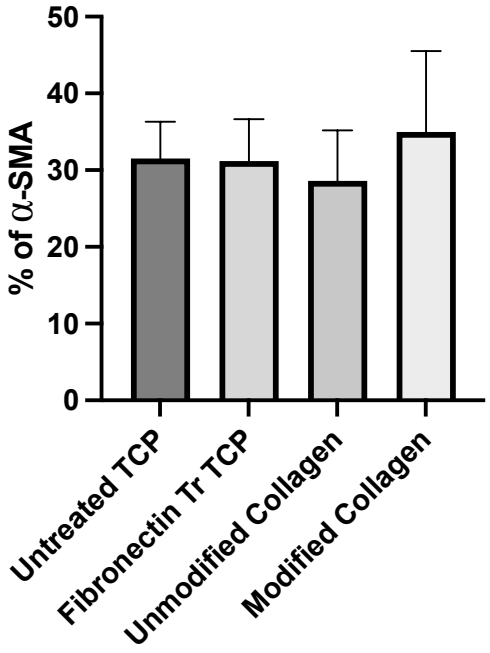

Figure 13 The percentage of cells expressing α-SMA when cultured on a TCP, a TCP treated with fibronectin, an unmodified collagen plate and an MG-modified collagen plate (n=5).

A One-Way ANOVA indicated there was no statistical significance between conditions (Fig. 13; p=0.5881). Tukey's multiple comparisons test indicated no statistical significance between pairs of each condition.

3.3.2 Adhesion Assay

An adhesion assay was run to assess cardiac fibroblasts response to MG exposure and test the hypothesis that cardiac fibroblast adhesion will increase when cultured on an unmodified collagen plate versus an MG-modified collagen plate. Cardiac fibroblasts from five heart donors were used to for this assay (n=5).

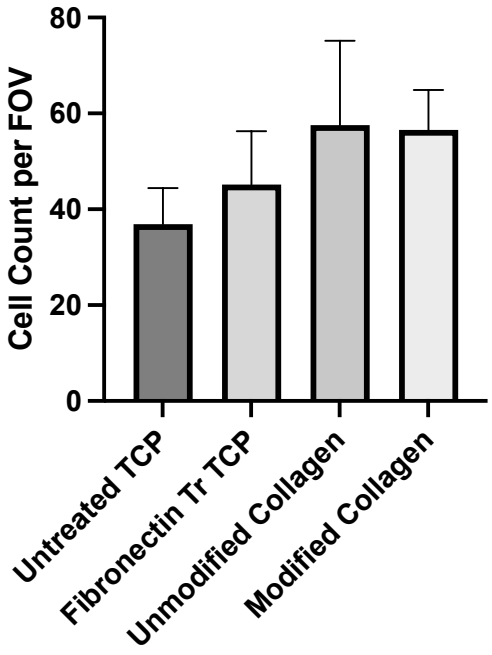

Figure 14 The cell count per field of view when cultured on a TCP, a TCP treated with fibronectin, an unmodified collagen plate and an MG-modified collagen plate (n=5).

A One-Way ANOVA indicated there was no statistical significance between conditions (Fig. 14; p=0.6546). Tukey's multiple comparisons test indicated no statistical significance between pairs of each condition.

3.3.3 Cardiac Fibroblast ITGA11 Expression when Cultured on Unmodified or MG-Modified Collagen with and without fisetin

Representative images of ITGA11 expression in cardiac fibroblasts under each condition can be seen in Figure 15. The data was analyzed by comparing specific conditions with each other, as seen in Figure 16.

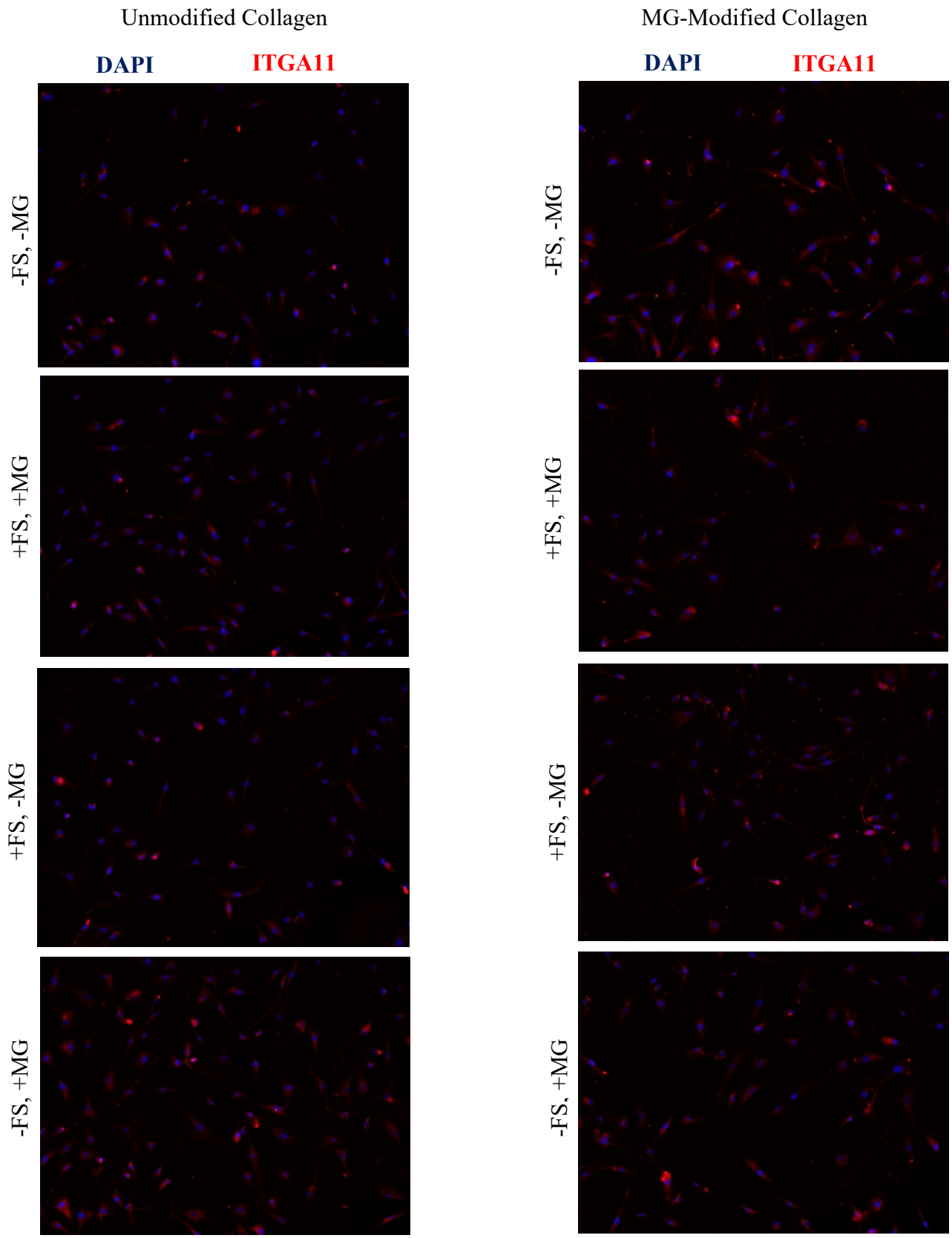

Figure 15 Representative images of cellular expression of ITGA11 when treated +/-MG and/or +/-FS and cultured on an unmodified or MG-modified collagen plate. Blue = DAPI, red = ITGA11.

3.3.3.1 Cardiac Fibroblast Expression of ITGA11 on Modified VS Unmodified Type I Collagen Plate

An ICC assay was conducted to test the hypothesis that cardiac fibroblasts cultured on an MG-modified collagen plate will express an elevated level of ITGA11 compared to cardiac fibroblasts cultured on unmodified collagen plates. The three measurements considered are the % of ITGA11 expressed, the mean intensity per cell and the integrated density per cell. Cardiac fibroblasts from three heart donors were used to for these assays (n=3).

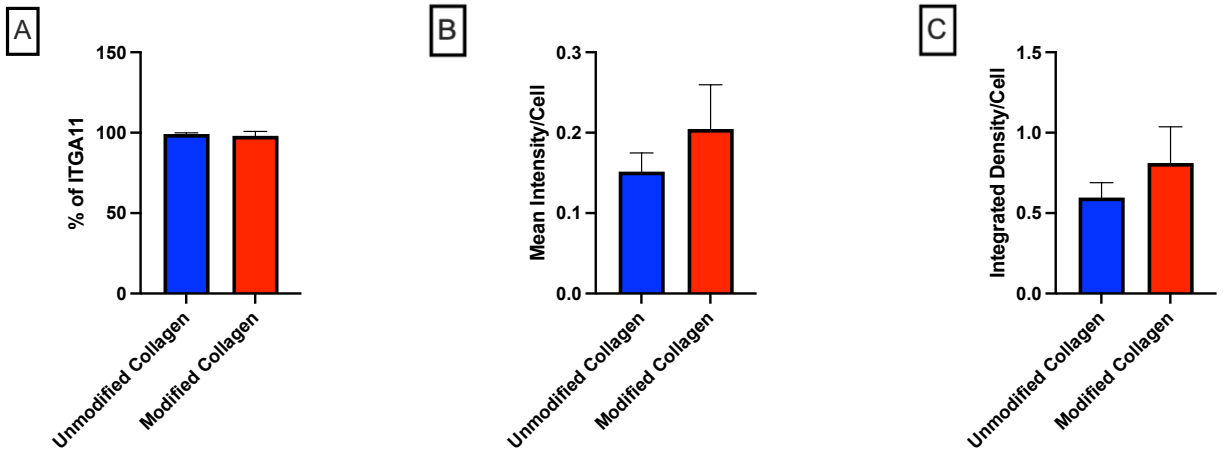

Figure 16 The percentage of cells expressing ITGA11 when cultured on unmodified collagen vs MG-modified collagen (A). The mean intensity of ITGA11 expression per cell when cultured on unmodified collagen vs MG-modified collagen (B). The integrated density of ITGA11 expression per cell when cultured on unmodified collagen vs MG-modified collagen (C) (n=3).

An unpaired T-test was used for each comparison. There was no significant difference of the % of ITGA11 (Fig. 16; df=4, t(df)=0.6737, p=0.5374). Additionally, no significant differences were found between mean intensity per cell (df=4, t(df)=1.537, p=0.1992) or integrated intensity per cell (df=4, t(df)=1.531, p=0.2004).

3.3.3.2 Cardiac Fibroblast Expression of ITGA11 on Modified VS Unmodified Type I Collagen Plate +/- Fisetin treated cells

An ICC assay was conducted to assess the effect of fisetin on cardiac fibroblasts cultured on an MG-modified collagen plate. The hypothesis tested was that cardiac fibroblasts treated with fisetin and cultured on an MG-modified collagen plate will express a lower level of ITGA11 than cardiac fibroblasts without fisetin cultured on an MG-modified collagen plate.

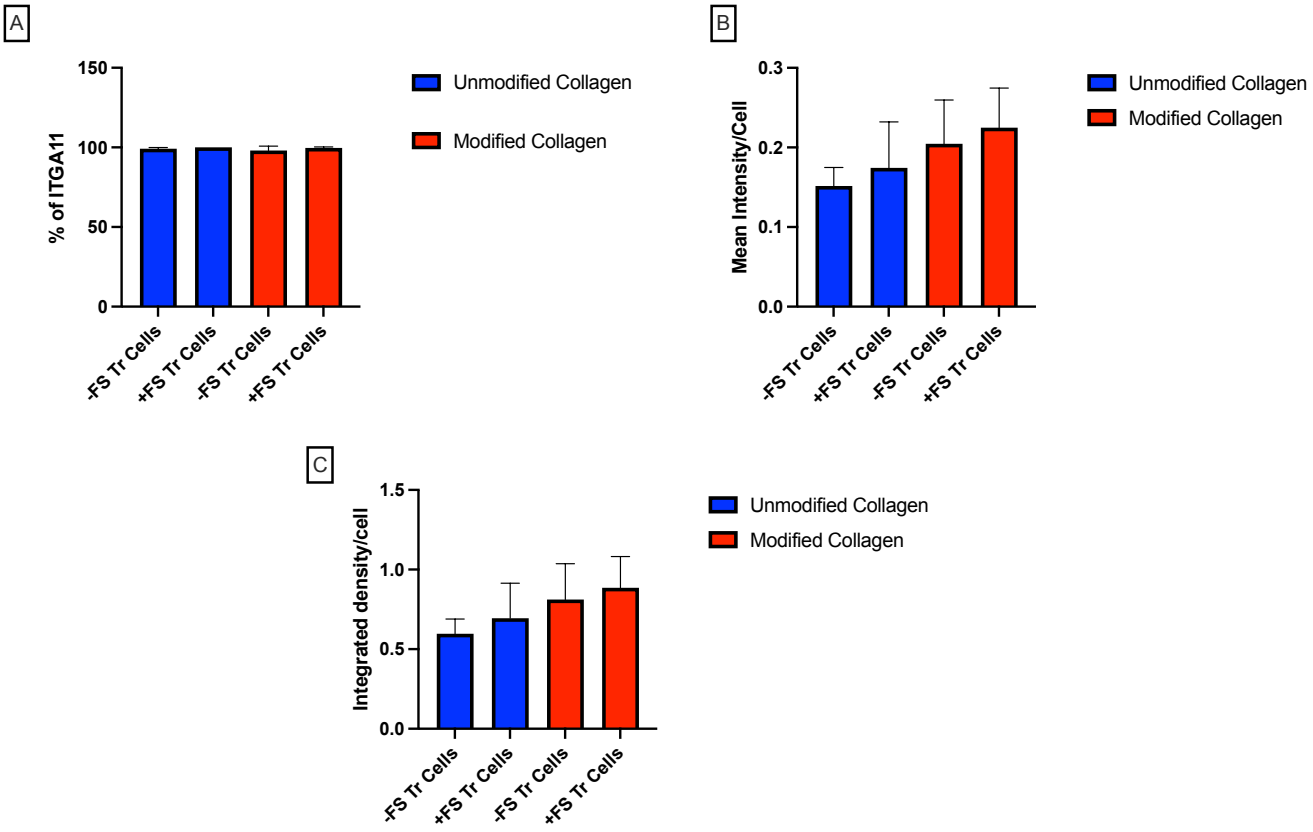

Figure 17 The percentage of cells expressing ITGA11 (A), the mean intensity of ITGA11 expression per cell (B) and the integrated intensity of ITGA11 expression per cell (C) when cultured on unmodified collagen +/- FS or MG-modified collagen +/- FS (n=3).

The three measurements considered are the % of ITGA11 expressed, the mean intensity per cell and the integrated density per cell. Cardiac fibroblasts from three heart donors were used to for these assays (n=3).

A One-Way ANOVA indicated no statistical significance between of the % of ITGA11 expressed (Fig. 17; p=0.4272). No significant differences were found between mean intensity per cell (p=0.3289) or integrated density per cell (p=0.3298). Tukey's multiple comparisons test indicated no statistical significance between pairs of each condition.

3.3.3.3 Cardiac Fibroblast Expression of ITGA11 on Modified VS Unmodified Type I Collagen Plate +/- Fisetin and/or +/- MG treated cells

To test the hypothesis that cardiac fibroblasts treated with fisetin + MG will express a lower level of ITGA11 than cardiac fibroblasts treated with MG alone, an ICC assay was conducted.

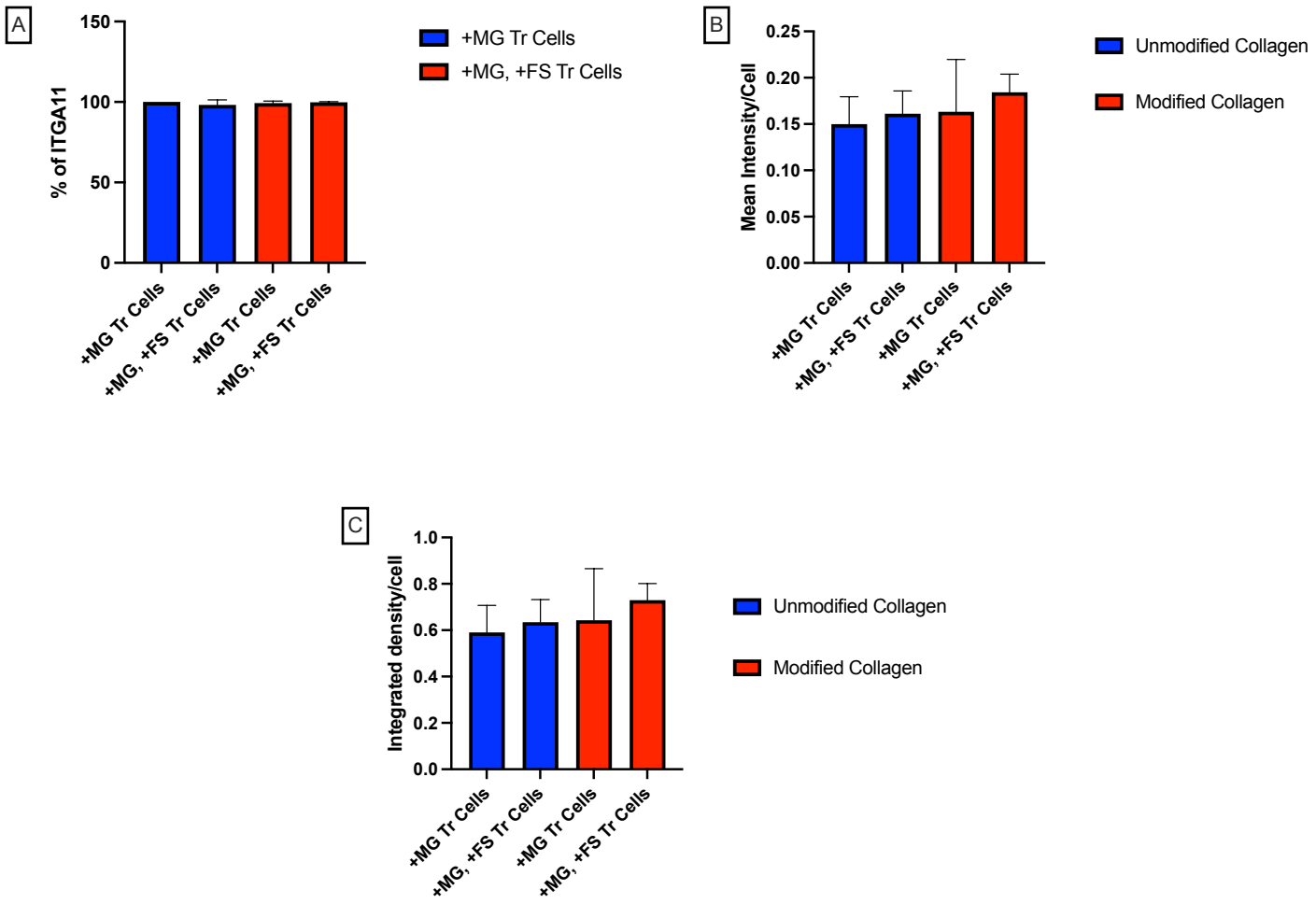

Figure 18 The percentage of cells expressing ITGA11 (A), the mean intensity of ITGA11 expression per cell (B) and the integrated intensity of ITGA11 expression per cell (C) when cultured on unmodified collagen, +/-FS and +/-MG or on MG-modified collagen, +/-FS and +/-MG (n=3).

The three measurements considered are the % of ITGA11 expressed, the mean intensity per cell and the integrated density per cell. Cardiac fibroblasts from three heart donors were used for these assays (n=3).

A One-Way ANOVA indicated no statistical significance between the % of ITGA11 expressed (Fig. 18; p=0.5929). No significant differences were found between mean intensity per cell (p=0.4864) or integrated density per cell (p=0.5224). Tukey's multiple comparisons test indicated no statistical significance between pairs of each condition.

4. Discussion

The alarming rate of post-MI mortality necessitates further research on strategies to mitigate infarction-related cardiac dysfunction. A major obstacle seen for patients recovering from an MI is the pathological development of fibrosis in the injured heart. This can have several detrimental effects, in particular ventricular remodelling. The mechanisms that contribute to the development of fibrosis post-MI are not fully understood, however substantial evidence indicates that it is in part due to the accumulation of MG that occurs after an MI. This raises two important questions. First, what are the effects that MG has on the application of collagen-based hydrogel therapy, specifically during the degradation process of the hydrogel? Second, how does MG accumulation affect the ECM and cardiac cells involved in repair? Addressing these questions will help determine the mechanisms behind the pathological fibrosis seen after an MI, and to gauge the hydrogel's therapeutic potential. Herein, the results will be discussed by addressing the two questions/objectives described above.

4.1 Objective 1 – Effect of MG on collagen hydrogel and its degradation

The first trial was executed to assess the degree of MG-mediated collagen glycation of the hydrogels. The hydrogels used for these studies were largely composed of rat-tail collagen. Rat tail collagen is a suitable choice for testing collagen-based hydrogel therapies for two main reasons. For one, rat tail collagen gels with sufficient rapidity allowing for a its homogenous distribution upon injection (*Collagen Type I, Rat Tail | 3D Cell Culture Gels & Coating*). Additionally, rat tail collagen hydrogels create an *in-vivo* like environment (*Collagen Type I, Rat Tail | 3D Cell Culture Gels & Coating*).

Research has indicated that there are cytotoxic effects of MG after 25 uM of exposure (Eren Cimenci et al., 2022). For assays which involved treating fibroblast-loaded hydrogels with MG, the concentration had to be sufficiently high to modify the collagen, while preventing high cytotoxicity. To determine which MG concentrations would be appropriate for specific assays, a mass spectrometry analysis was conducted. The concentration of MG-H1, an abundant MG-derived AGE (Blackburn et al., 2017), was detected to evaluate the degree of collagen glycation among samples.

As speculated, based on the mass spectrometry analysis, there was collagen glycation that occurred in the hydrogels incubated in 25 uM, 100 uM and 1 mM of MG. The statistically significant differences between samples indicated a positive correlation between concentration of MG and the level of MG-H1 formation. The results from this assay were directly applied throughout this study. An MG concentration of 25 uM was used when fibroblasts were treated directly with MG over a

prolonged period. 1 mM of MG was used to modify collagen when fibroblasts were not present, and 100 uM was used if the fibroblast-loaded hydrogels were incubated in MG only overnight.

An important consideration for biomaterials is the environment they will be exposed to. In this case, the hydrogel therapy would be interacting with the ECM and cardiac cells following its injection to the myocardium post-MI. The functionality of the hydrogels may therefore be affected by elevated levels of MG present in the post-MI environment. Additionally, a key characteristic of hydrogels is their ability to degrade at an optimal rate. It must remain in the intended area long enough to support healthy tissue formation, but must also be degraded and metabolized appropriately (O'Brien, 2011). Accordingly, the degradation rate of MG-modified hydrogels was assessed. Contrary to the hypothesis, no statistically significant differences were found between the degradation rate of MG-modified hydrogels and unmodified hydrogels.

As a potent glycating agent, it was suspected that the MG would modify collagen and interfere with the hydrogel degradation process, decreasing the rate at which the hydrogel degrades. The first assay, using a Collagenase I solution, was conducted as a preliminary assessment of degradation rates. Type 1 Collagenase is a matrix metalloproteinase (MMP), called MMP-1, which is an endopeptidase that can contribute to both healthy and pathological tissue remodelling (Pardo & Selman, 2005). Human fibroblast collagenase (MMP-1) is largely responsible for collagen turnover in cardiac ECM and was therefore used to degrade the hydrogels (Pardo & Selman, 2005). The second and third assay used primary cardiac fibroblasts isolated from human heart donors. This offered a more representational assessment of the hydrogel digestion rates. Specifically, the

cells were mixed directly into the hydrogel to simulate the in vivo interaction between fibroblasts and their surrounding collagenous extracellular matrix.

In all the assays, a control was run concurrently, allowing the hydrogels to degrade using PBS alone. On average, the hydrogels in the control PBS conditions took much longer, taking approximately 38 days to fully degrade. In contrast, with the inclusion of fibroblasts in the gel, complete degradation occurred in 8-12 days, indicating that the fibroblasts effectively accelerated degradation of the hydrogel. An observation noted during the trials using fibroblasts as the degradation mechanism was that on day 4, there was an increase in weight. This temporary increase in weight coincided with a reduction in the structural integrity of the hydrogel, causing more media retention.

The consistency in results among the assays demonstrating very similar degradation rates between conditions, was unexpected. One possible explanation for these results is that the cleavage site during Type I Collagenase degradation is different from where MG-modifications take place. MG targets lysine and arginine residues to produce MG-derived AGEs, whereas MMP-1, the fibroblast collagenase which would participate in the hydrogel degradation, cleaves fibrillar collagens at a specific glycine-isoleucine or glycine-leucine bond (Blackburn et al., 2017; Lima & Baynes, 2013; Williams & Olsen, 2009). If the site of MG-modification is different from the collagenase cleavage site, it may suggest there is no interference, therefore normal degradation would occur regardless of the MG modifications.

Another potential reason for these results is the type of reaction that both MG and EDC/NHS participate in. Collagen lysine residues are involved in both carbodiimide-mediated crosslinking and in some MG glycation reactions. The EDC/NHS crosslinking of the hydrogel was completed prior to MG exposure during these assays. In EDC/NHS crosslinking, the carboxylate moiety on one amino acid side chain reacts with a primary amine on an adjacent lysine residue (Bax et al., 2017). This would render the lysine residue unavailable for MG modifications, leaving only arginine available to react with. This could, perhaps, reduce the production of MG-derived AGEs, limiting their influence on the hydrogel's physical characteristics, and therefore their influence on hydrogel degradation.

Since we had expected the hydrogel degradation rate to be different between MG-modified and unmodified hydrogels, we expected that the degradation products of MG-modified and unmodified hydrogels would also differ. The degradation product of the hydrogel is an important characteristic to consider since degradation products of collagen have been shown to possess bioactivity and contribute to wound healing (Lindsey et al., 2015). To determine if there was a difference between degradation products of MG-modified and unmodified hydrogels, an LDS-PAGE was run. Unfortunately, the results from the procedure were ineffective in addressing this. In columns loaded with degraded unmodified hydrogel product, degraded MG-modified hydrogel product, denatured degraded unmodified hydrogel product, and denatured degraded MG-modified hydrogel product, a single band was seen near the top of the gel. This suggests that the protein samples did not separate while running the gel, preventing an analysis on hydrogel degradation products.

The crosslinking by EDC/NHS encourages polymerization of the triple helix collagen fibrils into fibers, forming the three-dimensional collagen gel (YANG, 2012). During MMP-1 degradation, the collagen fibrils are cut at specific sites, resulting in smaller segments of the triple helix tertiary structure (Xiao et al., 2010). While the primary structure is no longer intact, the secondary and tertiary structure remains.

When running an LDS-PAGE, it is important to denature the secondary and tertiary structure of the protein to allow protein separation based on charge/mass ratio. The samples were heated and treated in LDS, with the sampling buffer intended to disrupt the disulfide bonds of the triple helix. Insufficient denaturation may have resulted in the large band near the inlet of the well. This result was observed in wells loaded with LDS-prepared MMP-1-degraded hydrogels.

To try and denature the collagen further, samples were heated prior to the addition of LDS. Again, this did not appear to sufficiently linearize the degraded collagen fibrils, presenting a single band near the inlet of the wells.

It is unclear why the protein samples did not seem to denature sufficiently; however, one possible explanation is that the bonds formed between adjacent triple helix segments remained, as collagen is sticky in nature, causing the segments to clump together, forming a band. Another theory is that the LDS was ineffective in breaking the disulfide bonds sufficiently after degradation by MMP-1, which would also result in the single band.

To evaluate the effects of the degradation products of MG-modified and unmodified hydrogels on cardiac fibroblasts, an ICC was conducted. The expression of both αSMA and ITGA11 was assessed. Studies have shown that the expression of ITGA11 increases when fibroblasts adhere to glycated collagen (Talior-Volodarsky et al., 2015). The ITGA11 expression contributes to myofibroblast formation and sequential fibrosis seen in diabetic patients (Talior-Volodarsky et al., 2012). Based on the literature, expression of ITGA11 is stimulated by glycated collagen, however, it remains unclear whether ITGA11 expression is stimulated by the degradation product of an MG-modified hydrogel. The α-SMA was used as a myofibroblasts marker. α-SMA is a contractile protein produced by myofibroblasts to upregulate fibroblast contractility and prompt ECM protein synthesis (Humeres & Frangogiannis, 2019). Detection of the differentiation of cardiac fibroblasts into myofibroblasts is a promising cellular target suggesting fibrotic tissue development. It was hypothesized that there would be an increase in both α-SMA and ITGA11 expression when fibroblasts were treated with the degradation products of MG-modified hydrogels, due to exposure to MG-H1 and other MG-derived AGEs.

The results found contradicted the hypothesis, indicating no significant difference between α-SMA and ITGA11 expression after exposure to MG-modified and unmodified hydrogel degradation products. The ITGA11 expression was very similar in all conditions, including the fibroblast growth medium control. The results for the expression of α-SMA, however, showed a trend for higher expression of α-SMA from the fibroblasts exposed to the degraded hydrogel, +/- MG modifications. Although no differences were found, a greater sample size may have been needed to obtain statistical significance. If this were the case, a possible explanation might be that the products of the degraded hydrogels triggered a stress response from the cells, initiating

differentiation. Moreover, the protein expression from fibroblasts treated with MG-modified and unmodified hydrogel degradation products were very similar, which could mean that the MG-derived AGEs, formed prior to fibroblast exposure, may not affect fibroblast differentiation. A subsequent assay to help elucidate the effects of hydrogel degradation products on cardiac fibroblasts and determine any effects on function, is a viability assay or an assessment of MMP production.

4.2 Objective 2 – Effect of MG-modified collagen on cardiac fibroblasts

It has been found that MG-modified collagen can disrupt cellular attachment capabilities, indicated by reduced adhesion of fibroblasts to MG-modified collagen (Alqahtani et al., 2021). A cell-ECM adhesion assay was conducted, using a Type 1 Collagen-coated plate to mimic the high levels of collagen found in the cardiac ECM. Based on recent evidence, it was hypothesized that MG-modified collagen would reduce fibroblast adhesion. An evaluation of the results, however, demonstrated discrepant findings from the literature. While there appeared to be greater adhesion of fibroblasts grown on the collagen +/-MG plates versus the TCP+/- fibronectin plates, there appeared to be very similar adhesion levels of fibroblasts between cells grown on MG-modified and unmodified collagen. These trends, however, remain speculative as the cell count per FOV did not have statistically significant differences between any conditions.

A study by Algahtani et al., indicating a reduction of fibroblast adhesion to MG-modified collagen, incubated the collagen-coated plate in 5-10X higher MG concentration for a prolonged period than what was used for this assay. Perhaps the notable difference that they found among conditions was due to the higher concentration and longer exposure to MG.

Using the same conditions as the adhesion assay, an ICC was conducted to investigate fibroblast differentiation into myofibroblasts when cultured on an MG-modified collagen plate. Literature suggests MG's involvement in the mechanisms behind fibrosis development post-MI. The hypothesized outcome of this assay was that there would be an elevated level of α-SMA, the hallmark of myofibroblasts, when exposed to MG-modified collagen.

Again, there was no statistical significance among conditions, contradicting the hypothesis. This could perhaps be attributed to insufficient MG collagen glycation, reducing the effects that would be otherwise seen. However, the mass spectrometry results do not support this possibility since MG modification of the collagen hydrogel was confirmed. Using a higher concentration of MG and exposing the collagen to the MG for a prolonged period would be an appropriate method to address this, however, this approach is limited because of the cytotoxic effects of MG. Another possible explanation is that the cells respond to MG rather than the MG-derived AGEs that they would have been exposed to during this assay. To assess this theory, MG could be used to treat fibroblasts and collagen concurrently, perhaps for a longer period.

To further elucidate the effects of MG-modified collagen on fibroblast activation into myofibroblasts, an additional ICC was conducted. Fibroblast expression of ITGA11 was assessed when exposed to unmodified and MG-modified collagen. It was reasoned that the MG-modified collagen would prompt fibroblast differentiation, which would be indicated by elevated levels of ITGA11. Fibroblasts cultured on MG-modified collagen does appear to express higher levels of

ITGA11 than when cultured on unmodified collagen, however, no statistical significance was found. A greater sample size would be important for validating this observation.

An analysis was also done to assess the ability of fisetin to reverse the effects of MG on fibroblasts. Again, ITGA11 expression was assessed to represent fibroblast differentiation. The role of fisetin as an MG scavenger led the hypothesis that cells treated with fisetin would express similar levels of ITGA11 to cells cultured on unmodified collagen without MG-cellular treatment. However, there was no significant difference between conditions. One pattern noted, however, was that the fisetin treated cells appear to have a slightly elevated expression of ITGA11. While this observation is largely speculative due to the small sample size, it may hint that fisetin cannot scavenge MG-derived AGEs, and is only an effective scavenger for free MG. More research must be conducted to support this, however if it were the case, that may guide appropriate administration time of a fisetin-loaded hydrogels after an MI, requiring more immediate administration of the therapy before MG-glycation occurs.

5. Conclusion

5.1 Overview

A detrimental sequela experienced by many MI patients is pathological fibrosis. This fibrotic development can lead to ventricular remodeling and hinder cardiac repair. The endogenous mechanisms that contribute to poor cardiac repair post-MI is not well understood.

Fibroblasts, an abundant cardiac cell representing 2/3 of the cardiac population, have been shown to play a key role in regulating cardiac properties (Camelliti et al., 2005). A principal function of fibroblasts is the maintenance of heart tissue through regulating the dynamic structure of ECM. Fibroblasts will secrete important factors, such as collagen, cytokines and MMP's, which work to maintain balance between the synthesis and degradation of cardiac ECM (Aránguiz-Urroz et al., 2011). Fibroblast's role in ECM turnover makes them an important cell to consider when trying to establish the mechanisms behind post-MI fibrotic tissue development. Many *in vitro* assays for the study were used. An *in vitro* model offers benefits for these preliminary studies, including better control over the chemical and physical environment. This allows the researcher to isolate the specific variables being assessed. A novel aspect of the cellular assays used in these studies is that they were cardiac cells isolated directly from human donor hearts. Using human cells rather than cells derived from other animals offers more translatable results and a more representational *in vitro* model of the human heart.

During post-MI recovery, pathological scarring frequently occurs due to ECM remodeling at the site of injury. Another characteristic found in the cardiac environment post-MI is the accumulation of the toxin, MG. The relationship between MG and fibrotic tissue development was assessed by observing how MG-modified collagen affects cardiac fibroblasts. Reviewing the results of assays addressing this suggests that MG-modified collagen elevates fibroblast differentiation, however, there is no statistical significance. Additionally, fisetin, an MG scavenger, does not seem to affect fibroblast differentiation when exposed to MG-derived AGEs. These studies should be further addressed using a larger sample size, and in an *in vivo* model.

The degradative process of a biomaterial is an important characteristic to explore. A hydrogel therapy requires the appropriate degradation kinematics and appropriate morphological and chemical degradation profiles for its specific application. The hydrogel must be retained at the site of cardiac injury to elicit its therapeutic effects and degrade without leaving harmful or toxic foreign materials that may prompt an autoimmune response. Additionally, the environment that the hydrogel will be delivered into should be considered. Post-MI modifications may alter the environment that the hydrogel will be in, affecting its functionality and interactions with the ECM and cardiac cells. The degradation rates also become important to consider if the hydrogel is used as a biomolecule-delivery system to enhance its therapeutic effects, such as a fisetin-loaded hydrogels. The period that the hydrogel will be retained in the myocardium may affect how long it will provide therapeutic relief. These are characteristics that must be optimized before clinical translation.

The results of the present study suggest that the MG accumulation seen post-MI would not affect the degradation of the hydrogels by cardiac fibroblasts. Moreover, these preliminary studies show no statistically significant differences between the level of fibroblast differentiation when treated with MG-modified and unmodified hydrogels. Also, it appears that the degradation product of an MG-modified hydrogel does not activate fibroblasts any more than that of an unmodified hydrogel; however, it was observed that there was a trend for less fibroblast differentiation in assays without any degraded hydrogel product. Additional trials should be conducted, specifically in an *in vivo* model, to better represent the post-MI environment.

5.2 Limitations

While some interesting information can be drawn from these trials, they were not without their limitations. The assays involving cardiac fibroblasts used a two-dimensional (2D) model for cell culture. While growing cells as a monolayer on flasks is a widely used strategy, offering simplicity and low-cost, it can put stress on the cells and alter their function and structure (Kapałczyńska et al., 2018). The 2D model does not mimic a tissues natural structure and lacks intercellular and 3D ECM interactions and signals that would otherwise help guide cellular functions and growth. Some of the collagen plates were coated with Collagen Type 1 to resemble the cardiac ECM environment more closely; however, it was still based in a 2D model. Using an *in-vivo* animal model, or 3D model for culturing cells, would be beneficial for future studies, providing more clinically translatable results.

Additionally, the sample size for the cell-based trials was small. Conducting experiments using a greater sample size would offered more reliable results, potentially providing more statistical significance. The heart donors used for fibroblast isolation was limited in number, however, $n>3$ for experiments would be helpful for a better understanding of the results.

5.3 Future Research

As discussed, future studies would benefit from a larger sample size to gather more robust results. Moreover, using *in vivo* models would be an important step to incorporate the other environmental components of the post-MI heart, providing more relevant and clinically translatable results. Another focus should be to continue hydrogel optimization. A major benefit of hydrogels is their potential to act as a cell- or biomolecule-delivery system to enhance its therapeutic properties.

Interesting studies are being conducted to take advantage of this feature, such as incorporating nanoparticles or biomolecules like fisetin, into the hydrogel. Assessing how these loaded hydrogels confer therapeutic effects and determining their biocompatibility within the injured heart would be an important avenue to explore.

Post-MI recovery can take a significant toll on patients. Mitigating pathological modifications that occur after an infarction would have significant benefits by reducing patient burden and expediting their recovery. While a great deal of research is still required, the current trajectory of this research area holds promise for the development of treatments that will improve patient quality of life and ultimately, save lives.

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