Delivery of Biomaterials with Cytokines and Growth Factors for Myocardial Infarction Treatment

by

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DECLARATION

I hereby declare that the thesis is my original work, and it has been written by me in its entirety. I have duly acknowledged all the sources of information, which have been used in the thesis. This thesis has also not been submitted for any degree in any university previously.

Signature: 

ABSTRACT

One of the crucial and highly specialized organs in the human body is heart. It has limited regenerative and self-healing potential after the event of injury or illness. Therefore, heart transplantation is the primary treatment for end-stage cardiovascular disorders at the moment. Cardiovascular disease, particularly ischemic heart disease, continues to be one of the main causes of mortality worldwide. After a heart attack, the injured cardiomyocytes are lost and replaced with fibrotic scar tissue (Hashimoto et al., 2018). Cardiomyocyte loss has several consequences, for instance, the reduction of ventricular contraction, which triggers more cardiomyocyte loss, pathological cardiac dilatation, and ultimately heart failure. These series of events fall under the term "cardiac remodeling" (Tenreiro et al., 2021). The main objective of many treatments has been to prevent the gradual cardiac remodeling that leads to heart failure. Currently available effective treatments are medications, coronary artery bypass grafting, percutaneous coronary intervention and heart transplantation (Head et al., 2018)

This paper aims to describe a treatment protocol that includes the sequential administration of a composite cryogel with specific cytokines and growth factors. Firstly, the cryogel containing interleukin-10 (IL-10) was directly injected intramyocardially immediately after the induction of myocardial infarction, with the aim to target the acute inflammatory response. A second injection of cryogel loaded with vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2) was done on day 7 post-MI. The purpose of the second injection was to stimulate tissue regeneration and the formation of new blood vessels (neoangiogenesis).

In the cohort treated with Cryogel/GF, the study's results demonstrated significant myocardial tissue regeneration. Masson's Trichrome staining verified the echocardiographic findings, which showed decreased fibrotic areas and increased ejection fraction as well as fractional shortening. Therefore, the newly-developed chitosan-based cryogel contains anti-inflammatory and proangiogenic components, making this biomaterial an excellent cargo for the controlled release of therapeutic factors. Consequently, this will significantly improve tissue regeneration post-myocardial infarction.

Hypothesis: cryogel containing IL-10 cytokine and VEGF/FGF-2 growth factors delivered sequentially will improve cardiac regeneration after MI.

INTRODUCTION

I. Myocardial infarction. Definition and Prevalence

Cardiovascular diseases are known to be one of the most serious worldwide health concerns in the realm of noncommunicable diseases (Adhikary et al., 2022). Even though cardiovascular science and preventative medicine have developed many advantages in a past fifty years, the mortality and morbidity rates from cardiovascular diseases keep growing (Roth et al., 2017). According to studies (Alejandro Lerman et al., 2016, Nowbar et al., 2019) the main death cause globally is ischemic heart disease which accounts for almost 10 million deaths each year, and five million patients are affected by ventricular dysfunction caused by the ischemic cardiomyopathy followed by chronic heart failure. Moreover, myocardial infarction (MI), also known as heart attack, is the most common among other heart diseases. Hence, one out of two patients who have suffered from MI die within the next five years (Cambria et al., 2017). Talking about gender, cardiovascular diseases are more prevalent among men than women. For instance, in 2019, number of death among men was 9.6 million, whereas among women it is 8.9 million (*CV Disease Burden, Deaths Rising around the World*, 2020).

To be precise, MI is a pathological condition during which acute myocardial injury is observed. This occurs because part of the heart is blockeed and does not receive the oxygen-rich blood. Consequently, part of the heart muscle is damaged. The blood vessel is blocked usually because there are a lot of fatty deposits, also known as atherosclerosis and plaques in coronary arteries. This part of the heart will be damaged due to the lack of oxygen or even starts to die if the blood flow is not quickly restored (Ojha & Dhamoon, 2023).

The regenerative ability of the adult human heart is inherently restricted (Bergmann et al., 2009); therefore, the investigation of regenerative medicine therapies might be as a potential solution for repairing or replacing damaged hearts. Tissue regeneration is the complex phenomenon which involves a highly organized series of events, wherein cellular proliferation, differentiation, dedifferentiation, and coordinated morphogenic rearrangements occur in a sequential manner to restore tissue architecture as well as growth factors incorporation. Some sources report that unlike lower vertebrates such as newt and zebrafish, adult mammalian heart cells including human ones is incapable to restore damaged cardiac tissue (Gamba et al., 2014, Poss, 2002, Witman et al., 2011). Cardiac injury is associated with the loss of cardiac cells - cardiomyocytes. In response to damage mammalian heart fails to regenerate cardiomyocytes and therefore necrotic muscle substituted with scar tissue. That process consequently results in heart failure and death when the injury is quite severe (Porrello & Olson, 2014). Nevertheless, recently data has revealed that in adult mammalian heart tissues and in human ones, as well the cardiogenesis still occurs (Bergmann et al., 2015; Hashemzadeh et al., 2021). Therefore, myocardial tissue regeneration is highly promising therapeutic objective.

Such strategies as revascularization intervention as well as pharmacological approach is not an absolute cure (Jessup & Brozena, 2003). There is a scarcity of therapeutic options for the treatment of end-stage heart failure. However, it is important to note that heart transplantation remains the sole existing treatment for restoring the functionality of a compromised heart. Yet, according to Yacoub (2015) the main issue regarding heart transplantation is the gradual shortage of heart donors and increase in people in need.

Regarding the other types of treatments the main limitation here is the inability to regenerate damaged heart tissue as well as its function.

Ideally, after experiencing MI, the cardiac muscle goes through a healing process step by step. It begins with inflammation, followed by anti-inflammatory process, and ultimately shifting to tissue repair. Finally there is a scar tissue formed specifically in the area of infarct. Overall, there are two main steps of how does heart response to MI. First, proteases released by invading leukocytes degrade the extracellular matrix (ECM), tthis in turn is an important for removing dead cardiomyocytes. Subsequently, cardiac fibroblasts, the main contributors to ECM regeneration, primarily guide the formation of a new ECM that finally becomes a scar. In the process of transition from pro-inflammatory stage to anti-inflammatory and repair-focused ones, leukocytes play a crucial role. The balance between these stages determines the size and quality of scar, which in turn influences long-term post-MI consequences (Chalise et al., 2022).

Although inflammation is important for the process of healing and repair, it must be resolved in time to prevent worsening of the damage. Thus, anti-inflammatory cytokines take a stage in order to counterbalance the pro-inflammation and inducting the reparative phase after MI (Liu, Cheng, et al., 2020). Hence, in the repair phase, the activation of the anti-inflammatory cytokine IL-10 helps to reduce pro-inflammatory molecules, then tissue is cleared and healing process is promoted (Liberale et al., 2021).

II. Anatomy and Physiology of the Heart

Overview of the heart structure, cardiac tissue types and function

In adult mammals, the heart acts as the central connection between two types of circulations: systemic and pulmonary. It is around the size of one's clenched fist and possesses a distinctive four-chambered muscle structure. To be specific, the area that is adjacent to the apical part of the heart is anatomically separated into the left and right ventricles by the ventricular septum. Simultaneously, the atrial septum that is positioned above separates the left and right atria (Fig. 1). The observed architectural configuration of the heart reveals that both the left and right chambers possess a consistent reciprocating electrophysiological system. This system precisely regulates the body's hemodynamics through a sequence of significant axial motions (Enriquez et al., 2018). To be specific, during the diastolic phase of right atrium oxygen-depleted blood within the vena cava is actively drawn into the heart, thereafter, pushed into the lungs for the gas exchange by the muscular contractions of the right ventricle. Eventually the oxygen-rich blood is pumped into the left heart, facilitating the delivery of revival energy to the body (Fig. 1). Hence, the presence of muscle fibers in the cardiac tissue, particularly in the myocardium, which enables longitudinal, lateral, and oblique movement along the ventricular wall, plays a crucial role in facilitating efficient torsional motion in both clockwise and counterclockwise directions during systole and diastole. This motion is necessary to achieve optimal ejection and filling of the heart (Sanz et al., 2019, Bi et al., 2022). Figure 2 provides the information about delicate and distinct myocardial microstructure (Sommer et al., 2015). The outer myocardial tissue, a fibrous pericardium layer, locates the anatomical heart's position. Downward there are parietal and epicardial layers, that form the pericardial cavity, and its main function is secreting enough fluid and therefore to lubricate the

constantly moving organic constituents. The active myocardium layer- heart's core functional part- contains striated cardiomyocytes and blood vessels (Abdeltawab et al., 2020). The deepest layers known as endocardium and endothelial trabeculae enhance the heart's myocardium internal contractility and force generation. Additionally, the collagen-containing extracellular matrix (ECM) network maintains the heart's structural integrity, challenging artificial reshaping and supports its unique mode of operation. Such factors as coronary ischemic heart diseases (Spertus et al., 2020), severe congenital heart diseases (de Soysa et al., 2019), heart dysfunction due to bacterial or viral infections (Spellberg et al., 2020) cause heart's structural and functional changes. These changes affect one or more layers of myocardial tissue. According to Yasuhiro Shudo et al. (2019) heart transplantation is the preferred treatment for end-stage heart disease, but long-term survival is difficult due to donor scarcity and immune rejection. Thus, there is a need for extensive research into clinical transformation of advance tissue engineering hearts (Alonzo et al., 2019).

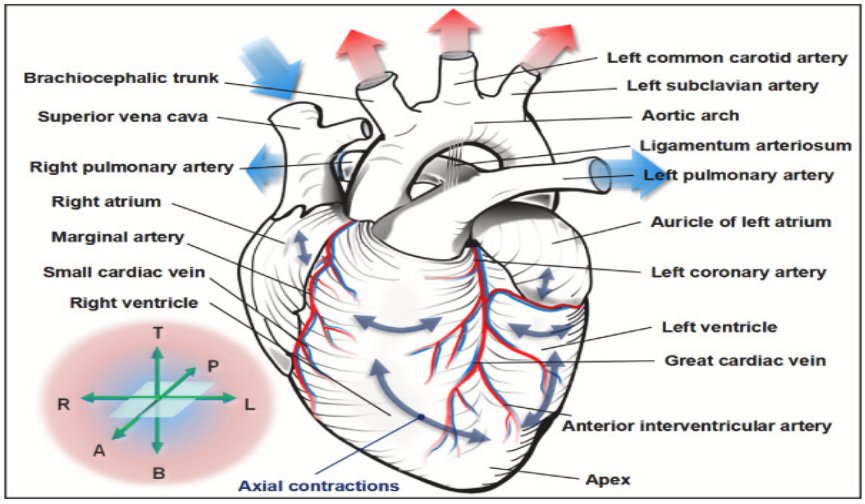

Figure 1. Anatomy of human heart.

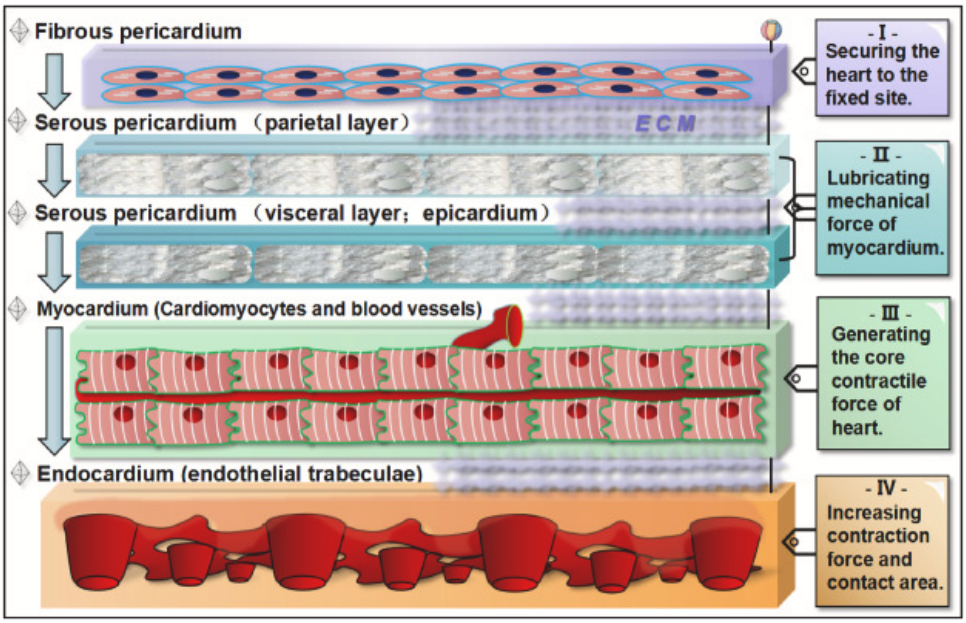

Figure 2. Cross-sectional representation of myocardial tissue layers.

Discussion of the inherent limited regenerative capacity of cardiac tissue

Cardiomyocytes are in a condition of terminal differentiation. However, there are variations in the cell cycle activity and proliferative potential of cardiomyocytes across different species and their life stages. Cardiomyocytes derived from specific animal species, including frogs, zebrafish and newts, exhibit the remarkable ability to undergo lifelong proliferation (Porrello & Olson, 2014). Contrarily, cardiomyocytes derived from adult mammals are in a state of persistent quiescence. Previous study of Sunny et al. (2022) demonstrated that regenerative capacity of cardiomyocytes following injury varies between 1-day-old and 1-week-old mouse heart. For instance, 1-day aged mouse heart is capable of regeneration over a period of three weeks after the injury; in contrast, 1-week-old mouse heart is unable to replace lost cardiomyocytes in response to injury. This research reveals a period of transition in the first week after birth when the mouse heart's regenerative capacity is diminished, that corresponds to the withdrawal of mammalian cardiomyocytes from the cell cycle. With the help of genetic lineage tracing, it was discovered that majority of 1-day-old mouse heart cardiomyocytes in the regenerated area originated from pre-existing cardiomyocytes. There is an opportunity to study the molecular mechanism of heart's transition from regenerative to a non-regenerative organ. Regarding the humans, a neonatal heart has regenerative capacity as seen by functional cardiac recovery following myocardial infarction (MI) (Haubner et al., 2016). Nevertheless, the turnover of cardiomyocytes is not sufficient to fully restore contraction functionality of injured heart. Thus, the development of cardiac regenerative therapies is a major milestone in repairing of cardiac function in individuals diagnosed with cardiovascular disorders.

III. Current treatments. Limitations

Medications

Different clinical studies and practice have demonstrated that medications play an important role by decresing the probability of mortality after MI. For instance, studies reported positive outcomes from the long-term use of such medications as oral antithrombotic agents, antiplatelet drugs, beta blockers, statins and angiotensin-converting-enzyme inhibitors (Strauss et al., 2021).

The mode of action of the antiplatelet drugs, main manager of MI, is to decres platelet aggregation in order to avoid clot formation in the coronary arteries. One of the type of antiplatelet agent is an aspirin. Although daily use of aspirin during the first month post-MI showed many advantages, this type of therapy still has some limitations. For instance, the constant intake of the aspirin leads to gastrointestinal toxicity. This in turn has another consequences such as risk of bleeding and ulceration (Arshad Muhammad Iqbal et al., 2022).

Percutaneous coronary intervention (PCI)

Percutaneous coronary intervention (PCI) is commonly used procedure to improve blood flow and prevent additional incidences of ischaemia. PCI is done under a minimal invasion, does not require surgery and aimed to eliminate constriction or blockage of the coronary artery. The main limitation is connected to such complications as injured heart arteries, bleeding, infections (Ahmad et al., 2020).

Coronary Artery Bypass Grafting

Coronary artery bypass grafting (CABG) is a medical procedure that is also used to improve blood flow to the heart. Unlike PCI, CABG is an invasive surgical procedure, that involves taking blood vessel from the chest, leg or arm of the patient and then engrafting it around the blocked coronary artery. The new blood vessel is called graft. Therefore, the blood flow is rerouted and cardiac muscles receive oxygen-rich blood and the probability of heart attack is decreased (Marghalani et al., 2023). Despite these benefits, there are some limitations. Compared to PCI, after the CABG, patients have longer period of recovery. Additionally, there are other risks such as bleeding, infections, and complications after anesthesia. Furthermore, after some time there is a chance that graft could become also narrowed or blocked, requiring repetition of the intervention (Bachar et al., 2023).

Heart transplantation

For individuals with end-stage heart failure following MI, heart transplantation remains the best therapeutic option (Tanveer et al., 2023). The surgery involves removing the patient's damaged heart and replacing it with a healthy donor one. Heart transplantation has its own set of drawbacks and difficulties, even if it can increase survival rates and quality of life. A major problem with heart transplantation is the lack of donor hearts, which in turn causes long waiting periods. Moreover, recipients must take immunosuppressive drugs for the rest of their lives after the surgery in order to prevent transplant rejection (Lauerer et al., 2016).

Overall, all these methods has common limitations, they are unable to regenerate damaged heart tissue and fully reestablish its functionality.

IV. Biomaterials

A biomaterial is a substance, either biological or synthesized one, that is specifically designed for medical purposes by interacting with biological systems (National Institute of Biomedical Imaging and Bioengineering, 2009). There are several important charachteristics that biomaterial has to possess. They are biocompatibility, biodegradability and bioactivity. A biomaterial is considered biocompatible if it can perform desired functions without causing any undesirable toxic effects, while also inducing an appropriate response in surrounding host environtment (Cohn et al., 2017). Property of biomaterial to be broken down under physiological conditions into non-toxic simple components is called biodegradability (Cohn et al., 2017). By the end of degradation, these small products are either absorbed or excreted by the body, guaranteeing safety and diminishing any adverse effects.

Based on the composition, there are two main categories of biomaterials. First one is natural such as collagen, hyaluronic acid, gelatin, laminin. Second one is synthetic such as polymers, ceramics and metals. The most often used formulations of biomaterials include hydrogels, nanospheres and nanoparticles, nanofibrous structures (National Institute of Biomedical Imaging and Bioengineering, 2009).

Cryogels, also known as specific type of hydrogel, are synthetic polymeric structures with a high degree of porosity. Since cryogel has all crucial features such as biocompatibility, biodegradability and bioactivity, it shows promise for use in cardiac tissue regeneration. Cryogel is biocompatible with cardiomyocytes, supporting their differentiation and proliferation (Sultankulov et al., 2019). Cryogel could be presented in different forms such as column, bead, sphere and membrane. Particularly for this research cryogel was designed in the laboratory of Professor Saparov and used in the form of powder. There are four main components of the cryogel: chitosan (CHI), heparin (Hep), and polyvinyl alcohol (PVA), with the addition of a glutaraldehyde (GA) cross-linker.

The main benefit of utilizing biomaterials involve the targeted delivery of substances to a specific area of interest. Therefore, it avoids certain disadvantages associated with systemic administration, such as increased risk of toxicity, allergies, or reduced efficacy due to lower therapeutic doses reaching the affected area or being distributed all over the body (Gaharwar et al., 2020).

MATERIALS AND METHODS

1. Preparation of the composite cryogel.

Preparation of cryogels was based on method described by Sultankulov et al., 2019a. In short, a 2% weight/volume solution of 0.4 g Chitosan (Sigma, United States) was dissolved in 20 ml 1% volume/volume acetic acid (Millipore, United States) and incubated on a hot plate at 50°C with a magnetic stirrer (500 rpm) for 1-2 hours. While a 1g of 5% w/v solution of poly (vinyl) alcohol (PVA, Sigma, United States) was prepared by dissolving it in 20 ml of deionized H_2O (dH_2O) using a magnetic stirrer (300rpm) on a heating plate for 1-2 hours at 90°C. 5 mg of heparin was dissolved in 5 ml of dH_2O. To prepare the composite cryogel following process was obeyed. Specifically, 5 ml of precooled PVA, 10 ml of chitosan, 5 ml of precooled heparin and 0.5% v/v of precooled glutaraldehyde (GA) we added one by one and mixed by magnetic stirrer.

After GA was added, the resulting mixture was promptly divided into precooled syringes of 3 ml each and placed in a −12°C water bath. The cryogels were then incubated for 12-24 h overnight. Subsequently, the cryogels were thawed, washed with water for 30 min using 50 ml tubes and shaker, and incubated one more time with 50 mM sodium borohydride ($NaBH_4$) solution on a benchtop shaker for 3 h. This step is required to neutralize the effect of GA (Migneault et al., 2004; Ivanov and Ljunggren, 2019). After the removal of excess water, the cryogels were frozen at -20°C for 1-2 hrs and then undergone freeze/drying process, also known as lyophilization, for a overnight using an LTE Scientific lyophilizer (United Kingdom). The dried cryogel samples were then stored at room temperature in a sealed tube until they were ready for use.

2. Preparing the solution with incorporated Factors IL-10 and VEGF/FGF into the cryogel.

First, dried cryogel particles were prepared by sequentially passing the dried chitosan cryogel through sieves with 100 um, 75 um and 50 um mesh pores. Then a 0.333 g of particles was taken and dissolved in 1 ml of 70% ethanol for 20 minutes in in a 1.5 ml Eppendorf tube. All bellow mentioned handlings were accomplished under sterile conditions in a cell culture hood. Then centrifuged for 1 min 1100 rpm (VWR Mega Star 600/600R Centrifuges, Bench Top, Ventilated/Refrigerated). After that EtOH was taken out and added 1 ml PBS. Centrifuged for 1 min 1100 rpm, then take out PBS. Repeated this cycle one more time. Next, added 1 ug/ml of IL-10 + 1 ml PBS to the cryogel. Similar procedure is done for VEGF/FGF. 10 ug/ml of VEGF/FGF and 1 ml PBS were added to the cryogel. After addition of factors to the cryogel the solutions were incubated at 37°C for 2 hrs 900 rpm each time in order for microparticles to properly load cytokines and growth factors. Then centrifuged 1 min 1100 rpm, excess PBS taken out, new portion of sterile 1 ml PBS added to prepare the mixture with the concentration of 10 ug/30 ul. Stored at +4°C. Ready to use for injection.

3. Animals.

C57BL/6 mice (males, weight is around 20-25g, 6-12 weeks old) were obtained from The Jackson Laboratory (United stated of America). There was a total of 3 groups; sham (n4), control (n4), cryogel/IL-10/VEGF/FGF (n4). The use of mice was approved by the Institutional Animal Care and Use Committee (IUCAC) of Nazarbayev University autonomous organization of education (Astana, Kazakhstan). All the processes were carried out under aseptic conditions. Animals were kept in specialized cages with wood fiber bedding, water and natural-ingredient grains inside. Mice were kept under 12/12 dark-light cycle. Isoflurane (Piramal Enterprises, Ltd., Telangana, India) was used to anesthetize mice in order to minimize the pain level during the research. At the end of experiment, the mice were euthanized by lethal injection of pentobarbital and arterial hemorrhage, thereafter tissue samples from the wound were collected for further studies.

4. Ligation of LAD coronary artery MI mice models.

12 C57BL6 mice aged 6-12 weeks were used for the in vivo LAD ligation experiment. The use of mice was approved by the Institutional Animal Care and Use Committee (IUCAC) of Nazarbayev University. Myocardial infarction was artificially induced via left anterior descending (LAD) coronary artery ligation. For the LAD ligation mouse, first, mouse was anesthetized for 3-4 min using inhalation of 4% isoflurane and 100% oxygen 2 L/hr using an anasthesia induction system for small animals. Next, endotracheal intubation was done immideately. Following that, animal was attached to the MINIVENT ventilator system for mice at tidal volume of 220 μl/stroke and ventilation rate of 140 strokes/minute, 2 % isoflurane and 100 % oxygen. The next step was performing thoracotomy between 3^{rd} and 4^{th} ribs, 3^{rd} intercostal space, in the left side of the breast cage. In order to clearly see the heart, there was a need in its slight exposure via self-made retractors, avoiding touching heart and lungs. Then, pericardium was accurately removed via forceps. Under the ZEISS Stemi 508 stereo microscope LAD was found and visualized. LAD artery appeared as superficial bright red vessel from the auricle towards the apex of the heart. Then, utilizing 7-0 sized silk suture coronary artery was ligated 2 mm below the auricle of the left atrium. The decolorization of left ventricle below the ligature into the pale red was the evidence of successful occlusion. Further, after finishing the ligation, the chest wall including ribs, subcutaneous tissue and skin were closed using 4-0 suture. Isoflurane delivery was switched off, and the mouse was observed for the appearance of spontaneous breathing. Following that, mouse was accurately extubated and located into the cage.

5. Intramyocardial injection of cryogel with growth factors/cytokines into MI model из bioactive materials

For more clarity, it is important to notice that intramyocardial injection of cryogel/factors were done at two different time points. Cryogel/IL-10 (30 μL) was injected intramyocardially first. Injetion was done immidiately after the LAD ligation operation into the infarction border zone of the treatment group. Next, cryogel/VEGF (30 μL) is prepared for the second injection

done on day 7 post-MI. This ultrasound-guided closed-chest intramyocardial injection is done via 27-G needle. The fixation of the syringe was done by VisualSonics micromanipulator before the injection. The needle tip was aligned within the myocardium to deliver injections at the desired spot, all under the control of the ultrasonic field-of-view.

6. Histological analysis

First of all, for histological analysis it was necessary to euthanize mice by cervical dislocation. Then through the abdominal cavities hearts were extracted accurately. Using pre-cooled PBS hearts were washed 3 times. 4% paraformaldehyde solution (Abcam 37% Formalin) was used for fixation of the hearts and left for dry up for 24-48 hrs. Next, hearts were placed into separate tissue base molds that had been labeled in advance, and cryo-embedding medium (OCT) ”Sakura” was applied completely over them. These tissue molds with hearts were placed into cold and stored at -80C until they are ready for sectioning. After that, iced hearts had been placed in a cryotome cryostat (CryoStar™ NX70 Cryostat) at temperature -20C. hearts tissue samples were cut into sections with 5 μm thickness. That procedure was done on left ventricular region where infarction zone is predicted to be located. Then positively charged glass slides were labeled and used to place there heart sections. All slides are left for dry up until staining process.

In order to measure the scar size and the level of fibrosis, Carl Roth GmbH Masson’s Trichrome stain was applied. Then rinsed for 10 minutes under the tap water. Next, procedure included three staining solutions. Goldner’s Stain I (Ponceau-Fuchsin solution) applied on sections for 15 min, and then they were briefly rinsed under the running tap water for 1 min. After that, sectoins were left for 1 min in the Goldner’s Stain II (Phosphotungstic acid-Orange G solution) until decoloration of connective tissue, followed by rinsing slides under the tap running water for 30 sec. The next step included counterstaining sections for 5 min with Goldner’s Stain III (Light green SF yellowish solution). Repeating the washing process for 2 min after final staining. Slides were left for dehydration for 1 hr and and mounted with a mounting medium DPX.
Scar tissue is stained in green, whereas muscle fibers and cytoplasm are coloured in red.

7. Echocardiographic analysis

Tranthoracic echocardiography also known as heart ultrasound. ECG was conducted twice on mice that had undergone surgery, once during the first week and then again during the fourth week following MI. The procedure was carried out under 2% isoflurane anesthesia. Measurements of the left ventricles were obtained using M-mode and B-mode imaging techniques at the level of the papillary muscles. The VEVO 2100 Imaging System was utilized for these measurements. Subsequently, Vevo LAB software was employed to analyze the gathered data, which included parameters such as left ventricular ejection fraction (LVEF), fractional shortening (FS), systolic diameter (SD), and diastolic diameter (DD), following the completion of the final echocardiography session.

8. Statistical analysis

All groups met the criteria for normal distribution. Therefore, a one-way ANOVA test was used to calculate and compare the means of the groups for echocardiography, histological and ultrasound analysis. Tukey's post-hoc test was then conducted to compare the groups further. Our findings are presented as means and standard deviations, with significance determined at a level of $p < 0.05$. For statistical analysis and graph creation, we utilized GraphPad Prism software (version 10).

RESULTS

Composite cryogel characterization

In order to prepare composite chitosan-based cryogel the previously established protocol was used. The protocol was developed specifically in the NUSOM in the Laboratory of Dr. Arman Saparov. The cryogel mass was then grinded into the powder (Fig.3). In the powder form the size of microparticles was around 50-100 µm. Cryogel was kept under sterile conditions such as UV light and ethanol so that any contaminations are eliminated.

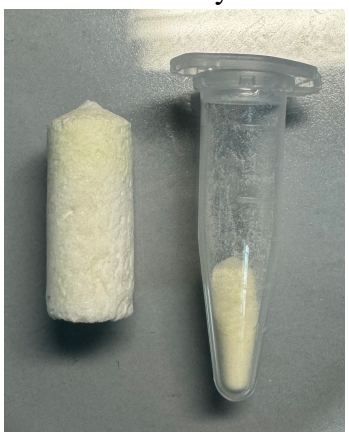

Figure 3. Two forms of prepared cryogel. Solid column form- left side. Powder microparticles form- right side.

Figure 4. Preparing the solution with incorporated factors IL-10 and VEGF/FGF into the cryogel.

LAD model of MI

Figure 5. Left image is an induction chamber used for delivering isoflurane to anesthesize mice for 3-5 minutes before surgery. Right image a small animals anesthesia equipment.

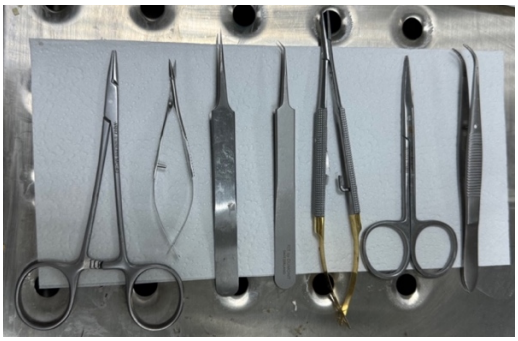

Figure 6. Set of scissors, forceps and needle holder used during the surgery.

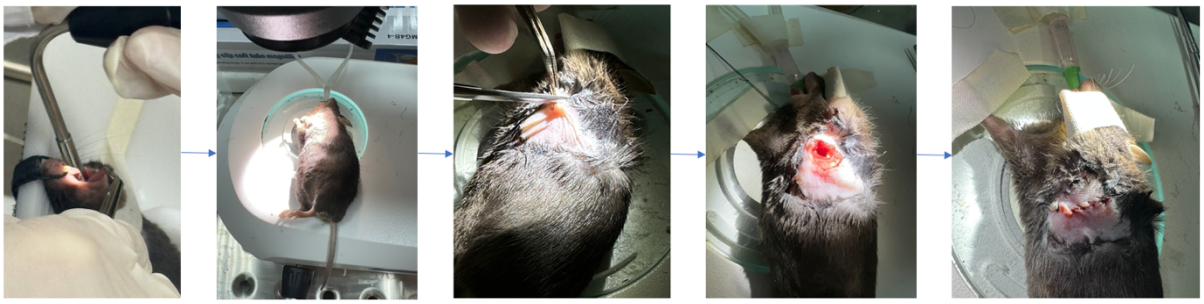

Figure 7. Full LAD artery ligation procedure. Intubation for 1 min, then linking mice to the oxygen supply system followed by skin incision, openning chest with retractors, LAD artery ligation itself and chest wall ligation.

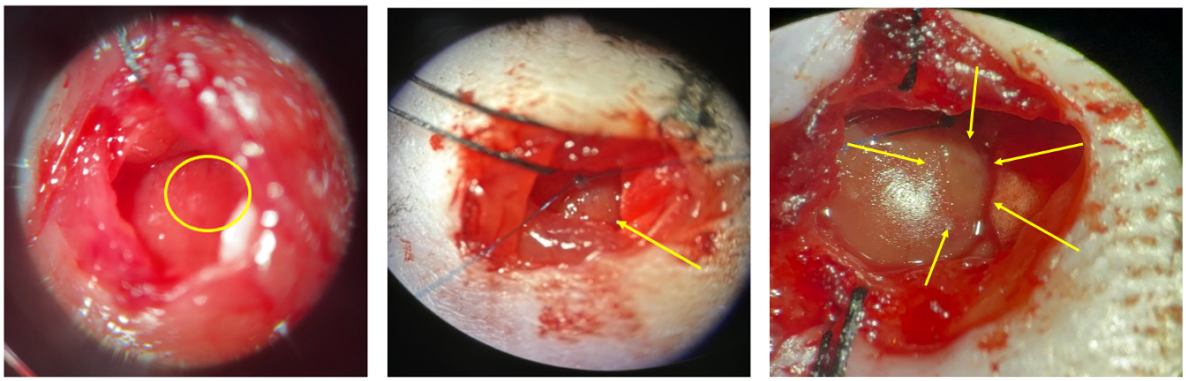

Figure 8. Images before and after LAD artery ligation done under ZEISS Stemi 508 stereo microscope. Left image- LAD artery area before ligation. Central and right images- cardiac region post-ligation; the needle and silk suture passed under the LAD, into the left ventricle.

Post-MI injections of the growth factors

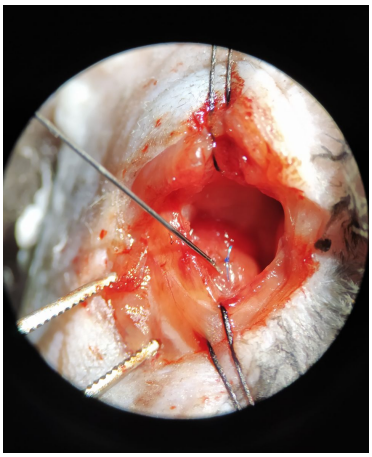

Figure 9. Intramyocardial injection of Cryo/(IL-10) into MI model
At the day of operation

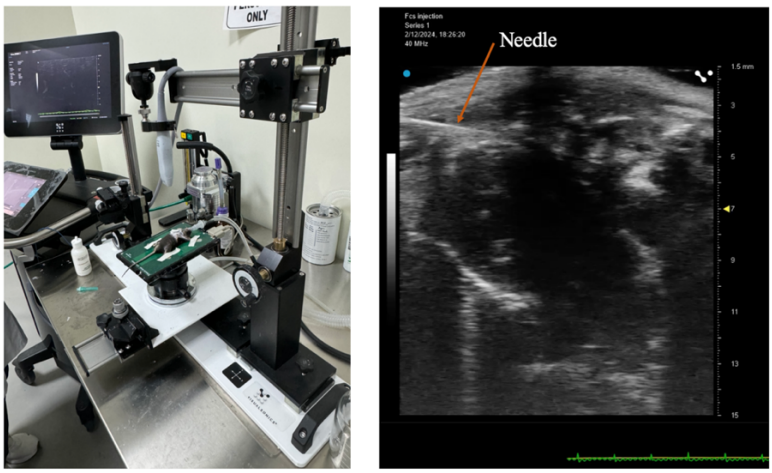

Figure 10. Left image represents the ultrasonographic system applied during the study. Ultrasound analysis conducted on week 1 and week 4 post operation for all groups of mice. Right image illustrates Ultrasound-guided closed-chest intramyocardial injection of Cryo/(VEGF/FgF2) done via 27-G needle on day 7 post-MI.

In vivo analysis of cardiac regeneration among treatment and control groups

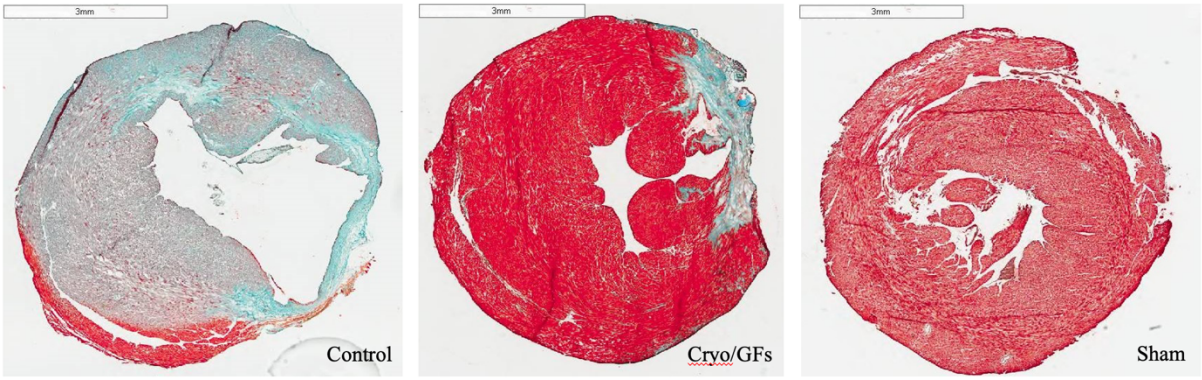

Figure 11. Analysis of cardiac regeneration across three groups. Myocardial sections from control, cryogel with GFs (Cryo/GFs) and sham groups were stained using Masson's Trichrome staining. Areas of fibrosis are stained in blue representing collagen fibers. Healthy heart tissue are stained in red. Scale bars =3 mm.

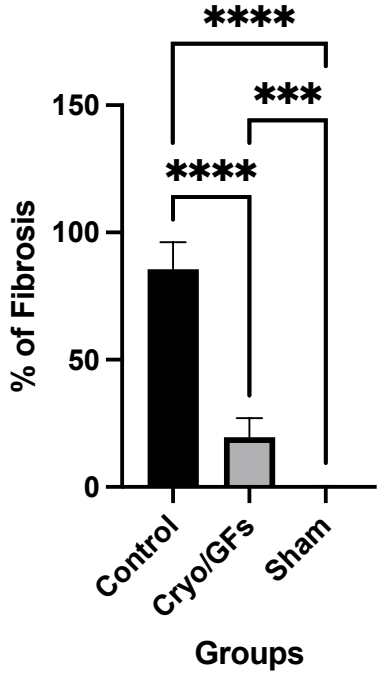

Figure 12. Percentage of fibrosis data obtained from histological sections was quantitatively analyzed using the ImageJ program. Y-axis of the bar graph illustrates the degree of fibrosis in three groups, presented as the mean ± SD, with statistical significance indicated (***p<0.001, ****p<0.0001). Bar graph was constructed using GraphPad.

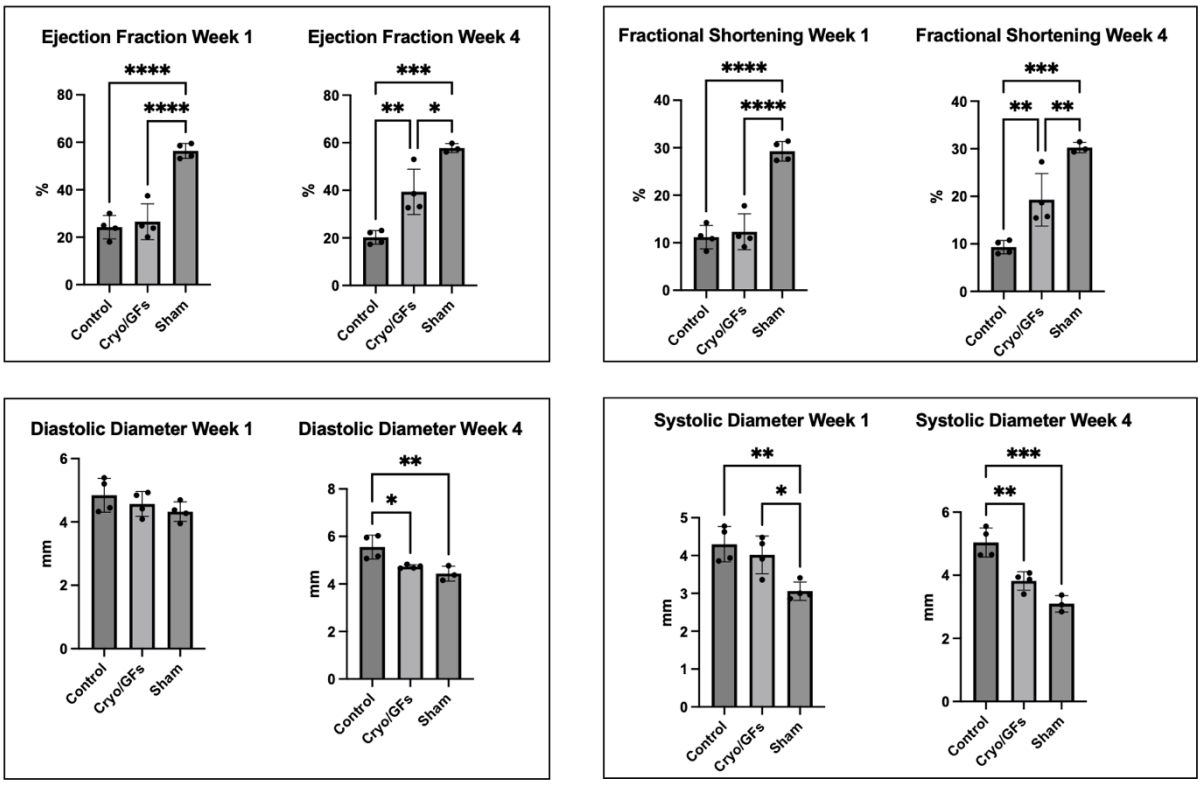

Figure 13. Cardiac function was measured using ultrasound analysis at Week 1 and Week 4 after treatment. Ejection fraction, fractional shortening, diastolic diameter, and systolic diameter were assesed. Data are represented as mean ± SD. Statistical data compare three groups at different time points, demonstrating changes in heart function, with statistical significance indicated (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

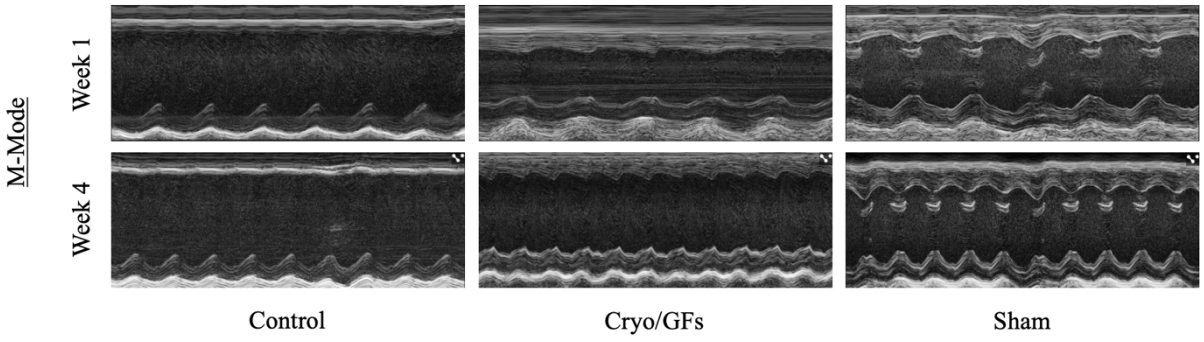

Figure 14. The ultrasound analysis provides illustrative M-Mode echocardiographic images that display the heart wall movements and chamber size for each group throughout the cardiac cycle. These images offer a visual representation of the quantitative ultrasound data, which shows the functionalality of the myocardium after the intervention.

DISCUSSION:

Composite cryogel characterization

Cryogel has to be biocompatible with tissue and biodegradable after use. This composite cryogel is resorbed and eliminateed from the body, does not cause and toxicity, facilitate natural regeneration of the heart tissue. Therefore, since cryogel has such an important qualities as biodegradability and biocompatibility, it is ideal option to be used in cardiac regenerative therapy.

Using cryogel synthesized in the laboratory of Dr. Saparov, cytokines and growth factors could be incorporated into the biomaterial matrices without any biochemical modifications maintaining biological activity of the microparticles. Therefore, the composition of the crygel was chosen to be CHI (chitosan), PVA (polyvnyl alcohol), GA (gluteeraldehyde) and Hep (heparine) in order to create cryogel that could effectively adsorb growth factors without any additional alterations.

The healing processes of an infarcted heart and wound have analogous mechanisms including formation of granulation tissue, neovascularization and inflammation control. The rationale behind this concept was that cryogel's capacity to sequentially deliver different cytokines and growth factors might be advantegous for post-MI regeneration process in myocardium. Thus, this could also potentially lead to a decrease in inflammation and an enhancement of angiogenesis and tissue remodelling.

The size range of microparticles between 50 and 100 μm was chosen to make sure that the particles aresmall enough to be injected yet still big and massive enough to keep their structural intact integrity.

According to IACUC Policy for Dose Volumes in Laboratory Animals, the recommended dose of intramuscular injection in mice is 30 ul (Cate, 2022). Additionally, analysing related research, research group in our lab established that the mass of cryogel powder should be 10 ug. Therefore, it was calculated 10 ug/ 30 ul, the concentration used per injection of IL-10 and VEGF/FGF.

LAD model of MI

In this research it was decided to use mice in order to establish *in vivo* model of MI and evaluate the efficacy of the treatment.

Before the starting of the operation, all the mice were anesthetized for 3-4 min under 4% isoflurane and 100% oxygen using a small animal anesthesia equipment to ensure that the mice is remained sedated without affecting animals' physiological stability (Fig. 5).

After these steps I intubated the mice to secure an airway (Fig. 7). The intubation was done quickly, for about half a minute, in order to minimize stress and avoid hypoxia. After that, mice were immediately connected to a oxygen supply ventilator system and kept in that way throughout the surgical procedure.

The main part of the MI induction surgery was the ligation of the left anterior descending (LAD) artery (Fig. 8). The procedure initiated with a left thoracotomy (exposure the heart), then identification and ligation of the LAD artery. Particularly, this step was done under magnification using microscope to assure precision and to prevent any unintended tissue damage. LAD ligation step took about 30-40 minutes. After the ligation was successfully completed, the ischemic area became pale pink colored (Fig. 8 yellow arrows), indicating that

the artery had been blocked and that MI is induced. By the end of every operation the chest wall has been sutured and mice were recovered in their cages.

Post-MI injections of the growth factors

The reason why injections were done sequentially is that I wanted to avoid overwhelming of the heart with too many injections at once. In this case there could be reduction of the efficacy of the treatment. Therefore, itt was decided that spacing will be beneficial and that is why injection of Cryo/(IL-10) was set on day of operation (Fig. 9), and injection of Cryo/(VEGF/FgF2) was set on day 7 post-MI (Fig. 10).

Injecting Cryo/(IL-10) at the day of operation would first reduce inflammation, and then injecting Cryo/(VEGF/FgF2) 7 days later might create an appropriate environment for the formation of new blood vessels, so that we can observe better survival.

In vivo analysis of cardiac regeneration among treatment and control groups

Histological analysis

For the histological analysis Masson's trichrome staining kit was used in order to measure the area of fibrosis on sections. So, at week 4 myocardial tissue sections from each group were stained and compared. On Fig. 11 healthy muscle fibers are colored in red, whereas the collagenous fibrotic tissue is colored in blue. Fibrosis is formed due to accumulation of fibrous connective tissue in injured area during the reparative process.

Fig. 11 represents the myocardial sections where an important reduction in fibrosis was achieved by the GF therapy. The decrease in fibrosis is specifically seen in the Cryo/GF group compared to the control group.

Further, in order to quantitatively calculate the percentage of fibrosis the histological sections were analysed using ImageJ program. Obtained data supported the visual assumptions (85.6±10 (Control) / 19.57±7.5 (Cryo/GFs) / 0.16±0.23 (Sham)) (Fig. 12).

Thus, according to the histological analysis, treatment with GFs was able to lessen the degree fibrosis developed after MI compared to the control group. Therefore, we might suggest improved cardiac tissue regeneration process.

Ultrasound Measurement of All Groups

Alongside with the histological analysis, the ultrasound measurements were done at Week 1 and Week 4 for all groups. Ejection Fraction (the percentage of blood the left ventricle pumps out with each contraction), Fractional Shortening (the percentage of the diameter the ventricle reduces during contraction), Diastolic Diameter (internal dimension of the ventricle when it is filled with blood) and Systolic Diameter (internal dimension of the ventricle after it has pumped out blood) are represented on Fig. 13, they are the key parameters that were measured. Measurements for other parameters, namely Cardiac Output, Stroke Volume, Diastolic Volume, and Systolic Volume were also performed and the data is provided in the appendix section. Remarkably, none of these parameters showed substantial differences among the groups at the end of Week 1. The reason for that is probably that heart function immediately after the therapy post-surgery is similar within all groups.

Although by the end of Week 1 data represent statistically significant positive changes, meaning the initiation of a pro-healing processes at the molecular and cellular levels, it still

does not mean that there are any noticable changes in overall cardiac function. However, noteworthy changes were observed in Cryo/GF group by the end of Week 4. Therefore, most prominent improvements in such parameters as ejection fraction, fractional shortening, and systolic and diastolic diameters were detected in the Cryo/GFs group.

The Cryo/GF group showed singnificant improvements in the ejection fraction and fractional shortening parameters, they are the crucial indicators of the heart's pumping efficiency. On the contrary, systolic and diastolic diameter parameters were reduced in Cryo/GFs group. This implies less ventricular remodeling, which is a common outcome of MI. Decreased ventricular dilation is correlated with a lower possibility of heart failure and improved overall cardiac function.

Fig. 14 represents an echocardiogram, providing one-dimensional image of the heart. The M-mode traces illustrate the movement of the heart walls during the cardiac cycle. As a result, the treated group at Week 4 showed more improved heart movements compared to the control.

Ultrasound data and ordinary one-way ANOVA multiple comparisons results with p-values are provided in the appendix. F-statistic and p-values as well results for post-hoc analysis are provided in the appendix section.

To conclude, during this study based on the histological, ultrasound, quantitative and echocardiographic analysis, it was observed a significant improvements in Cryo/GF treatment group compared to the control. These data place a promising therapeutic solutoin for the heart tissue regeneratiion post-MI.

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