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Alginate-gelatin-silk fibroin-containing patches improve cardiac function in an *in vivo* myocardial infarction murine model

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Abstract

Transplantation of patches based on alginate and gelatin biomaterials represents a promising approach for improving cardiac function *in vivo*. To enhance the long-term durability and mechanical properties of alginate-gelatin patches, silk fibroin was added to the formulation, and 3D printed alginate-gelatin-silk fibroin patches without cells were transplanted into an *in vivo* myocardial infarction murine model. The focus of this study is to evaluate whether the addition of silk fibroin to alginate-gelatin hydrogels can protect against the reduction in cardiac function after myocardial infarction. Mice were divided into four experimental groups: sham, myocardial infarction, myocardial infarction with the transplanted alginate-gelatin patch and myocardial infarction with the transplanted alginate-gelatin-silk fibroin patches. The ultrasound analyses confirmed that silk fibroin-containing patches increase the left ventricular ejection fraction % by 20% in infarcted mice. Furthermore, histological and transcriptomic analyses demonstrate that alginate-gelatin-silk fibroin patches stimulate tissue-repairing processes, reduce cardiac fibrosis, increase cardiac remodeling and inflammatory-regulating gene expression, and recapitulate cardiac function and tissue homeostasis in myocardial infarction mice. Altogether, our *in vivo* findings support the biofabrication of advanced cardiac tissues containing alginate-gelatin-silk fibroin for tissue engineering and regenerative medicine, representing a potential therapeutic approach for patients with myocardial infarction.

Keywords: Silk fibroin; Hydrogels; Biofabrication; Myocardial infarction

1. Introduction

Cardiovascular disease (CVD) remains the leading cause of global morbidity and mortality [1]. Myocardial infarction (MI) is one of the most prevalent CVDs and leads to several pathophysiological changes to the heart, such as inflammation, fibrosis and tissue remodeling [2]. Following an MI, the limited regenerative ability of the adult heart leads to the permanent loss of cardiomyocytes and impairment of cardiac function, resulting in heart failure (HF) [3, 4].

Macrophages play an important role in cardiac remodeling following injury, activating cardiac fibroblasts, which deposit extracellular matrix (ECM) components to form a collagenous tissue scar [5-8]. This process of attempted cardiac tissue repair is known as myocardial fibrosis [3, 9]. The scar reduces tissue elasticity and reduces the heart's contractile ability [5-7, 10, 11].

Three-dimensional (3D) bioprinting has emerged as a promising solution to facilitate the replacement of fibrotic tissue in HF patients [3, 12, 13], via the bioengineering of personalized cardiac tissues for patients who have had MI [3, 12, 13]. Among other tissues and organs, 3D printed tissues have shown promise for potential applications in humans [14-16]. In the heart, this cutting-edge technique involves the implantation of a 3D cardiac tissue patch onto the damaged area, aiming to enhance the regenerative capacity of the injured heart and restore function [17]. Moreover, cardiac patches can reverse the progression of pathology after MI, reducing the scar tissue area, promoting angiogenesis, reinforcing the mechanical properties of the tissue and preventing patients from developing HF [2, 18]. The convergence of advanced biomaterials and emerging bioprinting technologies has led to a new era of regenerative medicine, potentially revolutionizing the treatment of MI, and offering new avenues for cardiac repair and regeneration. For instance, alginate (Alg)-gelatin (Gel) (AlgGel) hydrogels have already been explored for cardiac tissue bioprinting, with high biocompatibility to embedded induced pluripotent stem cells (iPSCs)-derived cardiac spheroids (CSs) and approximating the complex cardiac microenvironment *in vitro* [12, 13, 17]. In particular, previous studies in our team have demonstrated that printed AlgGel patches have shown promise in protecting against the reduction in cardiac function in MI mice [12, 13, 17]. However, AlgGel patches have sub-optimal mechanical properties and tend to degrade in a physiological environment. This may limit their use for *in vivo* applications [9].

Silk-fibroin (SF) is a strong and stable polymer that has been extensively explored for vascular applications, both *in vitro* and *in vivo*. Produced by the *B. mori* silkworm, SF has emerged as a promising biomaterial for cardiac tissue engineering applications, owing to its exceptional

cytocompatibility, biodegradability, and mechanical properties ^[4, 9, 19]. In addition, given its tuneable properties and low immunogenic response in the human body, it has also shown the potential to provide an effective treatment for MI. Nevertheless, SF solutions require modifications for effective printing as a standalone component. AlgGel hydrogels have mechanically soft properties ^[9, 13, 20], therefore, SF has been incorporated into AlgGel hydrogels to improve the mechanical properties and printability of AlgGel hydrogels, and control cardiac muscle contractile function. In fact, our laboratory's *in vitro* results demonstrated that adding 1% (w/v) SF to 4%Alg-8%Gel hydrogels enhanced their elastic properties and exhibited improved contractile activity of CSs embedded in AlgGel hydrogels ^[9]. Thus, following our previous studies using AlgGel *in vivo* ^[17] and now in combination with SF ^[9], our *in vivo* testing focused on the use of 1% SF added to 4%Alg-8%Gel hydrogels.

This study's hypothesis is that the addition of 1% SF to AlgGel patches (AlgGelSF) may improve cardiac function *in vivo*. In particular, we evaluated the effect of AlgGelSF patches on cardiac function to identify whether the enhanced mechanical properties can improve cardiac function after MI (**Figure 1**). Additionally, we investigated the complex interaction between 3D printed SF-containing patches and myocardial tissue post-MI on cardiac fibrosis, cell survival and gene expression involved in cardiac tissue remodeling.

Here, we show the effects of AlgGelSF-containing patches implanted on the apex of the MI mice hearts (**Figure 1**) following a permanent left anterior descending (LAD) artery ligation surgical procedure on RAG-1 immunodeficient mice. These models, devoid of functional T- and B-lymphocytes, offer a controlled environment for studying the interaction between the implanted constructs and the host tissue without the confounding variables of an intact immune response ^[21]. Investigating the *in vivo* post-MI response to 3D printed SF-containing patches in RAG-1 immunodeficient mice not only enhances our understanding of the regenerative potential of such constructs but also contributes to crucial insights for future clinical translation. Overall, this research endeavors to develop advanced therapeutic strategies for MI patients by combining biomaterial science, bioprinting technology, and immunodeficient murine models.

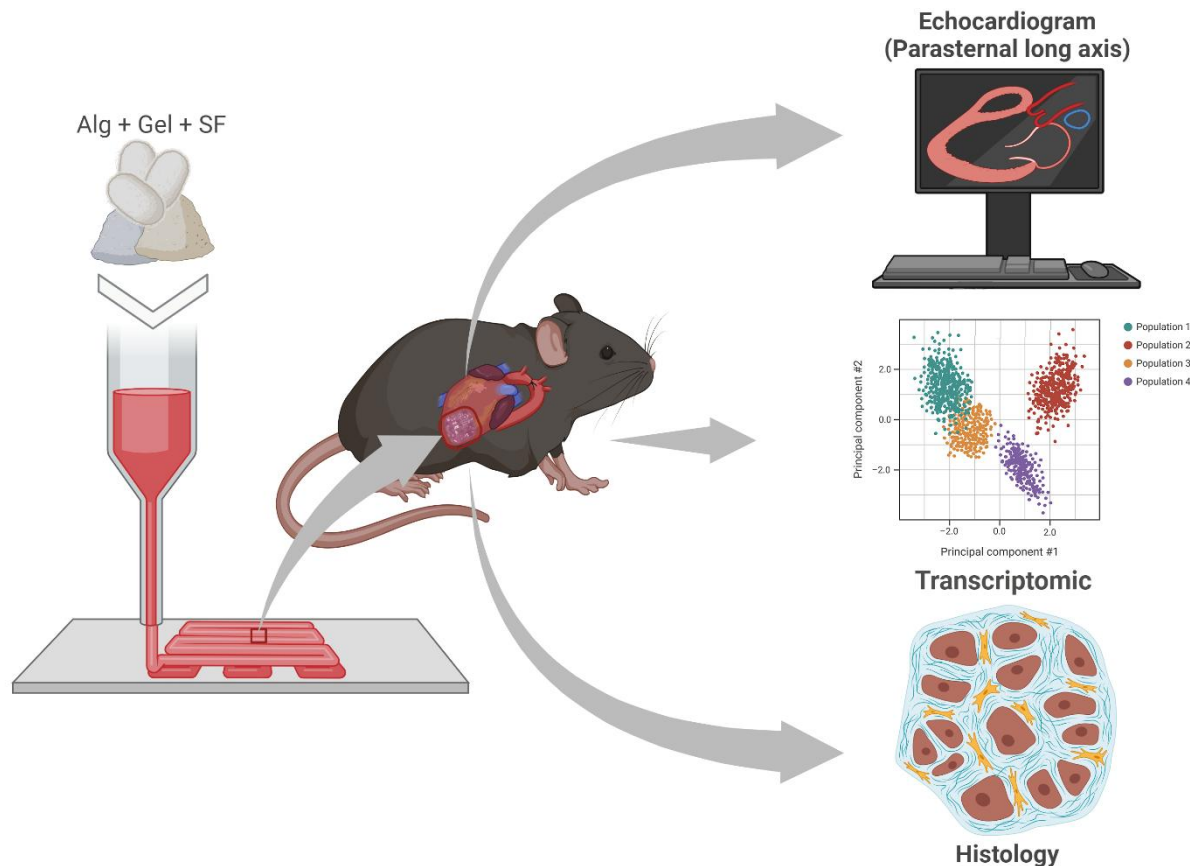


Figure 1. Schematic representation of the fabrication of SF-containing patches and the evaluation of AlgGelSF hydrogel's effects on cardiac function. AlgGelSF hydrogels were used to print cardiac patches, which were implanted into a mouse MI model. Echocardiography was used to evaluate the effect of the patch on EF%, while heart tissue sections were used to evaluate fibrotic and molecular changes via histological and transcriptomics analyses (Alg=alginate, Gel=gelatin, SF=silk fibroin).

2. Materials and methods

2.1. Materials

Bombyx mori silkworm cocoons were purchased from Sato Yama, Japan, while sodium alginate and gelatin from bovine skin were purchased from Sigma Aldrich, Darmstadt, Germany. Ketamine, Xylazine, and Atropine solutions for in vivo studies were purchased from Troy Laboratories, Glendenning, Australia.

2.2. SF isolation and SF-containing hybrid hydrogels preparation and crosslinking

SF protein was extracted as previously described to obtain an aqueous protein solution [22, 23]. Bombyx mori silkworm cocoons were degummed in 2 L 0.02 M sodium carbonate (Na_2CO_3 , Sigma Aldrich) solution at high temperature (90 °C–100 °C). Following rinsing and drying, degummed fibers were dissolved in 9.3 M lithium bromide (LiBr, Sigma Aldrich) solution at 60 °C (4 ml/1 g fibers). The last step involved a dialysis process, using a cellulose membrane (1 cm/3 ml SF solution) (SnakeSkin™ Dialysis Tubing, ThermoFisher Scientific), and a 3-cycle centrifugation (8700 rpm, 4 °C, 15 min each step). The SF aqueous solution obtained was 7%–9% (w/v) [9]. 4 g of sodium alginate (Alg, Sigma Aldrich) and 8 g of gelatin from bovine skin (Gel, Sigma Aldrich) powders were dissolved into 100 ml of Dulbecco's Modified Eagle's Medium-high glucose (DMEM, Sigma Aldrich), containing 1% (v/v) penicillin-streptomycin (Sigma Aldrich) and 1% (v/v) L-glutamine (Sigma Aldrich), at 60 °C under gentle rotation [9]. Based on the previous in vitro results [9], 1% (w/v) SF concentration was then mixed with 4% (w/v) Alg-8% (w/v) Gel hydrogels (AlgGel) and the final 4%Alg-8%Gel-1%SF (AlgGelSF) formulation was crosslinked using a 2% (w/v) calcium chloride (CaCl_2 , Sigma Aldrich) in Dulbecco's Phosphate Buffered Saline (DPBS, Sigma Aldrich) solution [9, 13].

2.3. 3D printing AlgGelSF-containing hybrid hydrogels and patches preparation for *in vivo* transplantation and tensile strength testing

AlgGelSF-containing inks were printed ($n \geq 6$) using the BIO X6 extrusion-based bioprinter (CELLINK, Gothenburg, Sweden). The 3D printing process protocol was optimized based on previous studies [9, 17]. BIO X6 cartridges were autoclaved and then placed with 22-gauge polypropylene nozzles under the biosafety cabinet and sterilized with a UV light cycle. The BIO X6 was sterilized through its built-in UV light. AlgGel and AlgGelSF solutions were warmed at 37°C before 3 ml of hydrogel was loaded into the bioprinter cartridge. Each SF hydrogel concentration was extruded through a 22-gauge size nozzle into 6-well plates. Samples were 3D printed with a rectilinear pattern as a lattice of 20 mm x 20 mm x 0.82 mm (width x length x height) with two layers of 0.41 mm, established by a Gcode created using the GPad code editor integrated in DNA Studio 4 software (CELLINK, Gothenburg, Sweden), with an infill grid density of 25% (Video S1). The printing parameters used were: pneumatic tool head set at 30°C, print bed

temperature set at 10°C, and extrusion speed set at 9 mm/sec. Pressure was adjusted during the printing process and ranged between 10 and 20 kPa. Following gelation and a one-hour crosslinking process, DMEM cell culture medium was added to each well, and patches were incubated at 37°C in 5% (v/v) CO₂ ^[9]. To avoid any disintegration of the patches, the movement of the plate was minimized. Cell culture medium was replaced carefully with a low-volume fresh medium every 2-3 days, and patches were cultured until the day of *in vivo* transplantation (up to 5 days). The 3D printed patches were cut into small pieces of 4 mm x 4 mm using a sterile surgical scalpel blade size 11 (*Sigma Aldrich*) immediately before the surgery. Each piece was placed on sterile aluminium foil and transferred to the procedure bench before transplantation.

Some additional patches were 3D printed for tensile strength testing and the printing protocol was optimized following the steps described above in this section. Samples were 3D printed into Petri dishes through a linear infill pattern, with two layers of 0.41 mm and with an infill density of 20%, established by a Gcode and edited through GPad code editor integrated in DNA Studio 4 software (*CELLINK*, Gothenburg, Sweden). AlgGel patches were printed as 60 x 50 x 0.82 (width x length x height), while AlgGel SF patches were printed as 60 mm x 20 mm x 0.82 mm. The pneumatic tool head was set at 28°C and with the temperature controller. The print bed temperature was set at 10°C and the extrusion speed was set at 5 mm/sec, while pressure was adjusted during the process between 35 and 45 kPa. Following crosslinking, AlgGel and AlgGelSF patches were used for strength testing (fatigue testing) using a Taihoya ATMS Dynamic Culture Technology System (*Capella Science*, Taiwan) (**Figures S1E-F**). Patches were carefully transferred to the Taihoya membranes by using spatulas; membranes with AlgGelSF patches were fixed at the instrument, and they were submerged in DMEM medium or DPBS. The instrument was set at 12 V, 1 Hz, 60 bpm and 20% dynamic stretch, to mimic the conditions of a healthy human heart (**Videos S2 and S3**). Patches were monitored for up to 24 hours.

2.4. Murine myocardial infarction and 3D printed patch transplantation

All procedures were approved by the Animal Ethics Committee at the University of Technology Sydney (ETH19-4338). The surgical procedures were performed according to our well-established protocol ^[17, 24] on B6.RAG-1 immunodeficient (B, T cell-depleted) male mice (Australian BioResources, Moss Vale, NSW, Australia), aged 9-10 weeks.

Mice were randomly divided into four experimental groups: a) Sham, b) MI, c) MI with the addition of the AlgGel patch, and d) MI with the addition of an AlgGelSF patch.

The anaesthetic solution comprised ketamine (40 mg/kg), xylazine (5 mg/kg), and atropine (0.15 mg/kg), with 100µl injected via intraperitoneal injection. Once the mouse was fully anaesthetized (after about 10 minutes), it was placed in a chamber inhaling 3 L/min isoflurane and 2 L/min oxygen. Following intubation, the mouse was carefully placed on a heated surface and connected to a PhysioSuite ventilator to keep the mouse constantly anaesthetized (isoflurane: 0-2 L/min; oxygen: 1-2 L/min) for the duration of the procedure. A few drops of betadine were applied to the mouse's chest and 100µl of buprenorphine (Temvet, 0.08 mg/kg) in 0.1 ml of 0.9% saline solution was administered via subcutaneous injection. All surgical procedures were performed using a microscope to aid in visualisation (Leica 651). Initially, a left thoracotomy was performed to access the mouse's heart. During the Sham procedure silk suture 7-0 (PERMA-HAND SILK, *ETHICON*) was passed under the left anterior descending artery (LAD artery) and it was tied loosely without occluding the artery, while MI was performed *via* permanent LAD ligation (occlusion of the LAD artery). Following the MI surgery, printed AlgGel and AlgGelSF-containing patches were immediately transplanted on the infarcted tissue area. Then, the muscles and skin layers of the chest were closed with a prolene suture 6-0 (PROLENE, *ETHICON*). Once the surgery was finished, isoflurane was stopped and the wound was wiped with betadine and ethanol. 100µl of Antisedan (atipamezole, 1 mg/kg), 100µl of Lasix (furosemide, 8 mg/kg), and 500µl of sodium chloride solution (0.9% saline solution) were injected *via* subcutaneous injections in different sites of the mouse. Bupivacaine (2 mg/ml in 0.9% saline solution) was also added onto the wound, and the mouse's eyes were lubricated with Poly-Tears Lubricating eye drops. Once the mouse began breathing independently, it was transferred to a chamber with 2 L/min of oxygen and monitored until fully recovered before being transferred to a cage with water and jelly food. During the postoperative period (the first week immediately after the procedure), mice were injected twice a day for the first 3 days and once a day the following 4 days with buprenorphine (0.08 mg/kg) through subcutaneous injections by a trained researcher (LV).

2.5. Mice echocardiography (heart ultrasound)

Echocardiography (ultrasound, US) was performed with a Vevo 3100 Preclinical Imaging System (*FUJIFILM VisualSonics*, Toronto, Canada) ^[25]. Mice were imaged at day 0 (baseline), day 14 (midpoint), and day 28 (endpoint) ^[17].

Before each ultrasound imaging, mice were weighed and then anaesthetized (isoflurane: 3 L/min, oxygen: 2L/min). Once asleep, mice were moved to the Fujifilm VisualSonics pre-warmed (37 °C) platform, where each mouse was placed supine and kept anaesthetized through a nose cone with 2 L/min isoflurane and 2 L/min oxygen. A small amount of conductive gel (Conductive Gel, *Livingstone*) was put on hands and feet of each mouse before being taped onto the four electrodes on the platform. Once the mouse was immobilized, shaving cream (Hair Removal Cream, *Nair*) was administered and spread on its chest. After a few minutes, the cream was removed using gauze swabs (*Livingstone*) and some US gel (Aquasonic Ultrasound Transmission Gel, *Parker*) was spread on the mouse's shaved chest. During the parasternal long-axis view, the transducer was rotated to an 11 o'clock position, and B modes of cardiac cycles were acquired. Throughout the echocardiography, the mouse's heart was maintained at >400 bpm (beats per minute), while cardiac cycles were recorded ^[17, 26].

US data related to the parasternal long-axis view were analyzed using VevoLAB 5.7.1 software (*FUJIFILM VisualSonics*, Toronto, Canada). US data was analyzed by outlining the diastolic and systolic cycles ($n \geq 4$) of the left ventricle using either the manual or the automatic (AutoLV) mode. A VisualSonics data specialist from Fujifilm validated the data.

2.6. Sample collection for histological, hypertrophy and transcriptomic analyses of the mouse hearts

At day 28 (endpoint) mice were euthanized and tissue samples were collected to perform histological and transcriptomic analyses ^[17]. Mice were first weighed and then kept under anaesthesia (isoflurane: 5 L/min, oxygen: 2 L/min) via a cone. The chest was opened and the heart exposed. Blood was aspirated with a 21-gauge needle and collected into tubes containing heparin. The heart was then collected and weighed. Transverse slices (1mm thick) of the left ventricle were obtained with a sharp blade. Heart slices were added to Eppendorf tubes with a 10% neutral buffer of formalin and kept at room temperature for histological analyses, or added to Eppendorf tubes and kept at -80°C in liquid nitrogen until RNA isolation for transcriptomic analyses.

Finally, the tibia was isolated, and its length was measured using a micrometer for hypertrophy analyses. Hypertrophy was evaluated by plotting the ratio of heart weight to body weight and/or the ratio of heart weight to tibia length ($n \geq 4$). Heart weights were normalized to body weights and tibia lengths to assess the increase of hypertrophic tissue in mice post-MI.

2.7. Histological analyses

2.7.1. Sample preparation and tissue processing

Heart tissue slices for histological staining were stored in 10% formalin neutral buffer for 10 days and then washed 3 times with 0.01% (v/v) sodium azide in PBS (PBSA). At the same time, histology cassettes were prepared and labelled with each mouse ID number. PBSA was removed, and each sample was placed between 2 sponges in each cassette. All cassettes containing the samples were submerged in 80% (v/v) ethanol for up to 1 week until processing. Tissue processing was performed through the automated tissue processor Thermo Scientific Excelsior ES Tissue Processor (*ThermoFisher Scientific*), which follows 6 cycles of ethanol, 3 cycles of xylene and 3 cycles of paraffin. Following tissue processing, all cassettes were opened and samples embedded in paraffin wax using the ThermoFisher Histostar Tissue Embedding Station (*ThermoFisher Scientific*), to be ready to be sectioned using the microtome Eprexia Microtome HM 325 (*ThermoFisher Scientific*). The microtome was set to cut 10 μm tissue sections; they were first unfolded in a tissue section bath set at 30-40°C and then placed on microscope glass slides ^[17].

2.7.2. De-paraffinisation, re-hydration and staining of tissue sections

The slides with the tissue sections were placed in a 55°C oven for ten minutes to melt the paraffin. Then, all the slides were placed in a rack to be de-paraffinized and rehydrated through several washes in xylene and ethanol. At the end of the de-paraffinisation process, the slides were washed in distilled water and placed in a rack.

Tissue sections were stained using Harris Hematoxylin Nuclear Stains (*Leica Biosystems*) and Eosin Secondary-Counter Stains (*Leica Biosystems*) to mark the nuclei of cardiac muscle cells and the ECM ^[27, 28], while others were stained with either Masson's Trichrome or Picro-Sirius Red Stain kits, to stain collagen and cardiac muscle fibers present in the cardiac tissue ^[29-32]. The hematoxylin and eosin (H&E) staining was performed through the automated machine Thermo

Shandon Varistain Gemini ES (automated tissue staining machine, *ThermoFisher Scientific*). Masson's Trichrome and Picro-Sirius Red staining were performed according to the manufacturer's protocols: Trichrome Stain Kit (Connective Tissue Stain, *Abcam*) and Picro-Sirius Red Stain Kit (Cardiac Muscle, *Abcam*), respectively ^[17].

2.7.3. Samples preparation for tissue imaging and analysis of fibrosis

At the end of each staining, all slides were placed in a chemical fume hood to dry, and then synthetic resin D.P.X. Neutral Mounting Medium (LABCHEM, *ThermoFisher Scientific*) was applied to each sample. Once mounted, glass coverslips were placed on top of each sample to let it dry before the edges were sealed with nail polish to prevent them from drying. Samples were then imaged through ZEN 2.6 software using Axio Scan.Z1 microscope (Zeiss) at 20X magnification, and each tissue staining was assessed through the plugin LabKit in ImageJ software (*NIH Fiji*). Samples were also imaged using an Olympus BX61 Upright fluorescence microscope at 20X magnification. The analysis of the fibrotic tissue percentage present in the four experimental mice tissues (n≥3) was performed in Excel on Masson's Trichrome and Picro-Sirius Red staining data and through the following formula:

$$\text{Percentage of fibrotic tissue (\%)} = \frac{\text{total fibrotic tissue area } (\mu\text{m}^2)}{\text{total tissue area } (\mu\text{m}^2)} \times 100$$

where 'total tissue area' represents both the fibrotic area and non-fibrotic (healthy) area of the tissue ^[17].

2.8. RNA-sequencing analyses

Cardiac apex cryosections of euthanized mice were processed for mRNA transcriptomic analysis according to the commercially available protocol of the RNeasy® Fibrous Tissue Mini Kit (*Qiagen*) ^[17]. Total RNA from each cardiac section was isolated by adding 14.3 M β-mercaptoethanol (β-ME) (*Sigma Aldrich*) to the samples and purified according to the manufacturer's instructions. The lysate concentration was then calculated using a NanoDrop Microvolume Spectrophotometer (*ThermoFisher Scientific*). Mice RNA samples (n≥3) were then independently processed by BGI Genomics (Batch no. INSP21000010163, Hong Kong, China), and RNA quality assessment, library preparation, and sequencing relying on DNBSEQ Eukaryotic Strand-specific mRNA

libraries sequencing technology were performed. Raw sequencing reads were filtered for adapters – reads, in which more than 25% of bases matched an adapter sequence. Reads matching the following five quality criteria were removed: 1) reads shorter than 150 bps, 2) reads with more than 0.1% N's, 3) reads with more than 50 bps of polyX's (A, T, G, C), 4) reads with more than 40% of bases having low quality (base quality < 20). The resultant high-quality reads were aligned to the mouse genome (mm10) through the software STAR (Spliced Transcripts Alignment to a Reference) ^[33] with standard parameters. An average of 23,603,986 reads per sample was mapped, achieving an average alignment rate of 98.52%. The featureCounts tool was used to quantify gene expression levels ^[34]. Genes with less than 100 reads across the samples were excluded, reducing the gene count from 26214 to 13058. Expression levels were normalized using the DeSeq2 software package ^[35] in the R statistical analysis software ^[36]. Genome-wide expression profiles were analyzed through MATLAB software's principal component analysis (PCA). Hierarchical clustering with complete linkage and Euclidean distance was performed using the pheatmap tool in the R statistical analysis software.

2.9. Statistical analyses

Results were plotted in GraphPad Prism™ (La Jolla, CA), and the statistical significance of the analyses was assessed using a Brown-Forsythe and Welch ANOVA test, together with a Dunnett's T3 multiple comparisons test. US data were analyzed using a two-way ANOVA test with Tukey's multiple comparisons test, and results were represented as either mean ± SEM or the median value. Fibrosis data are represented as mean ± SEM, while hypertrophy data are shown as median.

3. Results

3.1. AlgGelSF-containing patches are elastic and stretchable

To evaluate the potential protective effects of SF-containing patches against myocardial damage, these were implanted in an *in vivo* model of MI in mice (**Figure 1**). First, both patches containing AlgGel and AlgGelSF were printed as previously described (section 2.3, **Video S1**), cut into smaller square pieces (**Figure 2**), and measured using a micrometer to confirm their thickness (**Figures S2A** and **S2B**). As AlgGel hydrogels are less viscous compared to the AlgGelSF-containing ones, as demonstrated in our previous studies ^[9], AlgGel printed patches were slightly

thinner than patches containing SF, as shown in **Figures S2A** and **S2B**, respectively. However, both AlgGel and AlgGel patches were higher than the 2-layer height set on the 3D bioprinter software, and their sizes resulted in 4x4x2 mm. Both patch formulations were able to be manipulated and placed accurately over the heart (**Figures 3** and **Video S4**).

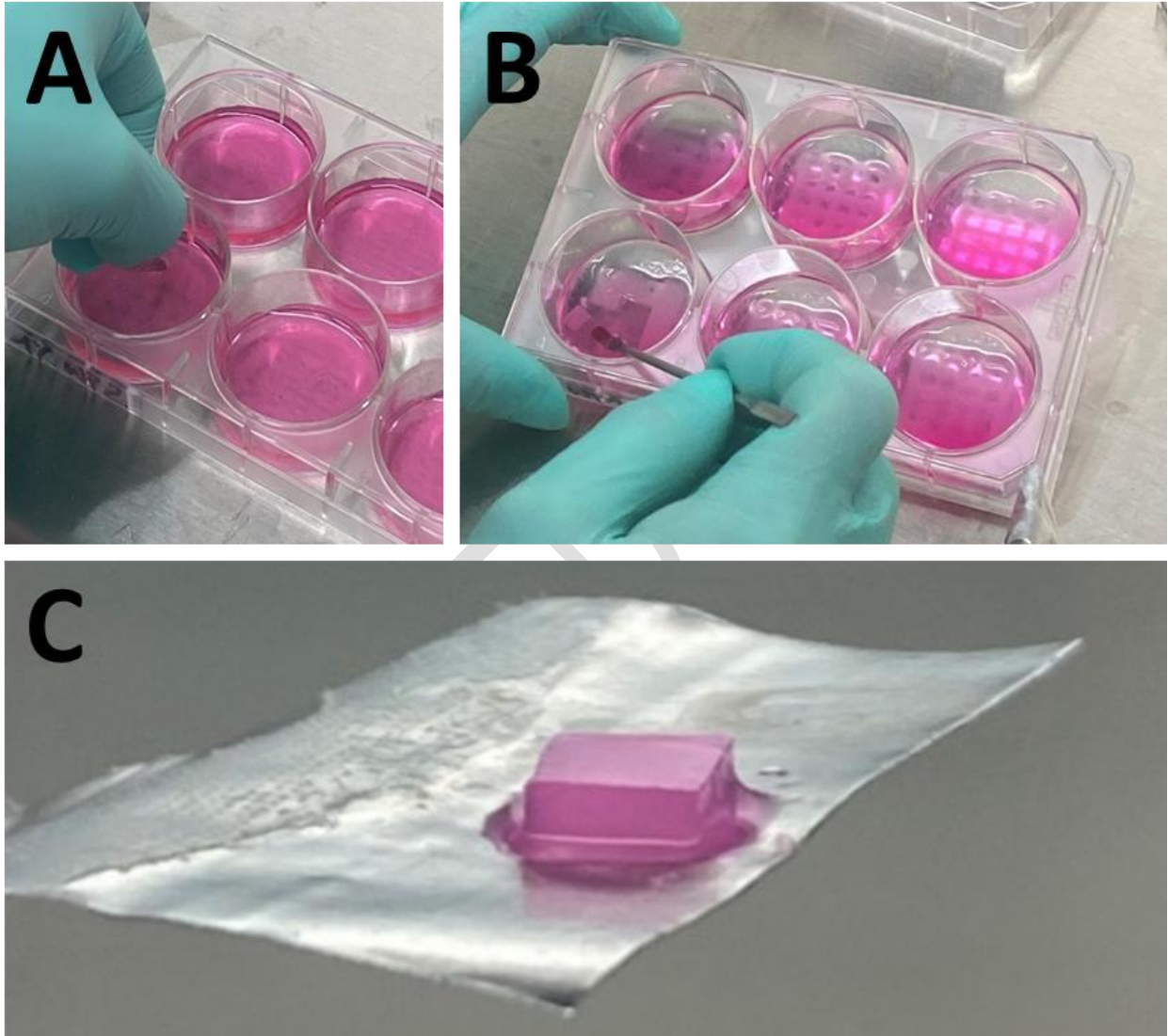


Figure 2. AlgGelSF patch preparation for in vivo transplantation. 3D printed AlgGel and AlgGelSF patches cultured in cell culture medium (**A**) and cut into smaller pieces (**B**). Cut patches on sterile aluminum foil prior to transfer (**C**) (Alg=alginate, Gel=Gelatin, SF=silk fibroin).

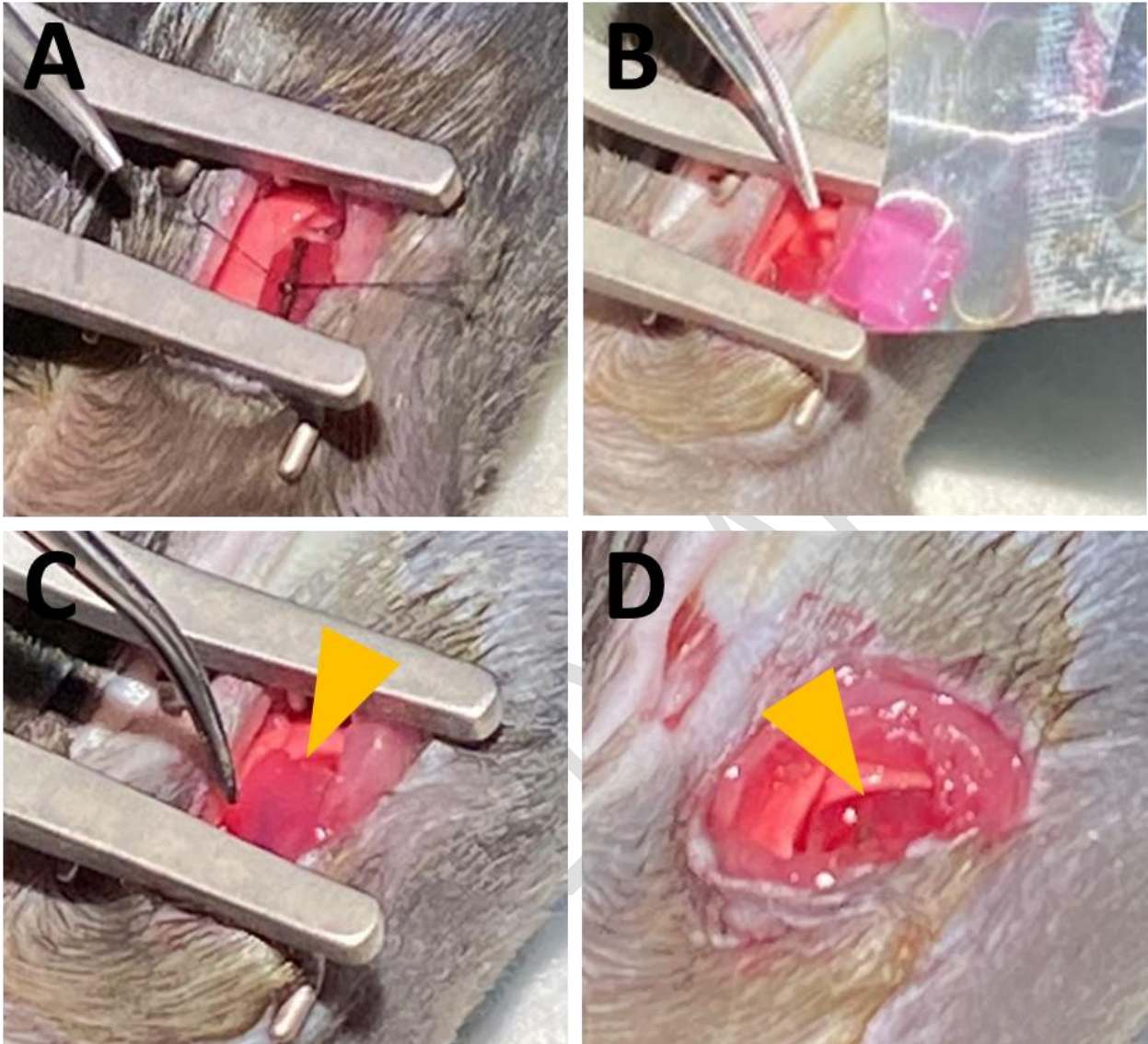


Figure 3. Myocardial infarction and 3D printed patches transplantation.

(A-D) The images represent a visual narrative of the sequential steps involved in the MI surgical procedure and subsequent transplantation of either AlgGel or AlgGelSF patches onto the MI site. Initially, the induction of MI is executed through the meticulous ligation of the left anterior descending (LAD) artery, accompanied by the strategic placement of a suture on the infarcted site (A). Subsequently, the 3D patch is transferred to the MI site (B) and precisely positioned onto the still-beating mouse heart using tweezers, as pointed by the yellow arrowhead (C). The successful integration of the 3D patch is highlighted as it remains securely in place on the MI site. Finally, the mouse's chest is carefully sewn to complete the procedure, ensuring the stability and permanence of the transplanted patch on the infarcted region, as pointed by the yellow arrowhead (D). These

images collectively illustrate the intricate and successful execution of the MI surgical procedure and 3D patch transplantation, showcasing the precision and efficacy of the experimental protocol (MI=myocardial infarction, Alg=alginate, Gel=Gelatin, SF=silk fibroin).

Patches incorporating SF exhibited superior handling characteristics compared to AlgGel ones (**Figures S1A-D**). When the 20x20x2 mm patches were lifted from the well plate using a thin spatula (**Figures S1A and S1C**), the AlgGel patches displayed a gel-like behavior, folding around the spatula (**Figures S1A**) and showing a tendency to break. Conversely, the AlgGel patches exhibited greater elasticity, demonstrating resistance to curvatures (**Figures S1C**) without succumbing to breakage. This flexibility was further evident when the patches were grasped and compressed between the fingers; after repeated movements and squeezing the AlgGel patches tended to break (**Figure S1B**), whereas the AlgGelSF patches remained intact (**Figure S1D**).

In addition, a test of the tensile strength of 0% and 1% SF patches was performed to mimic the mechanical stretch typical of the human heart tissue in the body under healthy human cardiac tissue conditions (**Figure S1E and S1F**). Over 24 hours, the stretchability of the AlgGelSF patch was monitored, and no rupture in the hydrogel structure was recorded (**Figure S1F**), while partial degradation was already visible in the AlgGel patch after 3h of testing (**Figure S1E and Video S2**).

3.2. AlgGelSF-containing patches protect against the reduction in cardiac function in infarcted mice

To evaluate the potential of AlgGelSF-containing patches to improve the LVEF% of myocardial infarcted mice, these were first implanted on the damaged area of mice hearts, and echocardiography was performed to obtain LVEF% data (**Figure 4 and Figure S3**). For each ultrasound measurement, both diastole (**Figure 4A**) and systole (**Figure 4B**) cycles were recorded. All experimental groups (*i.e.*, Sham, MI, MI with AlgGel patch transplanted, and MI with AlgGelSF-containing patch transplanted) did not present any significant difference in cardiac function at baseline as expected (**Figure 4C**). 14 days post-surgery (**Figure 4D**), mice with MI ($37.49\% \pm 4.64$) showed a reduced LVEF% of ~24% compared to Sham animals ($60.95\% \pm 3.52$), whereas mice with the transplanted AlgGel ($52.28\% \pm 4.56$) and AlgGelSF ($59.05\% \pm 3.60$)

patches showed an increase of LVEF% of ~15% and ~22% compared to the MI group, respectively. At the endpoint (28 days post-surgery, **Figure 4E**), MI mice with the transplanted AlgGelSF patch ($59.64\% \pm 3.72$) increased EF% by ~21% compared to MI mice ($39.07\% \pm 3.02$), while MI mice with the transplanted AlgGel patches ($49.51\% \pm 2.85$) increased by ~10% compared to the MI mice, with an overall difference in LVEF% by ~10% in MI mice transplanted AlgGelSF patches. Although there is a significant difference between mice with AlgGel patch and mice containing AlgGelSF patch, no difference is shown between Sham and the group with SF-containing patch, with SF patches closely mimicking cardiac function in Sham mice ($61.82\% \pm 4.18$) (**Figure 4F**).

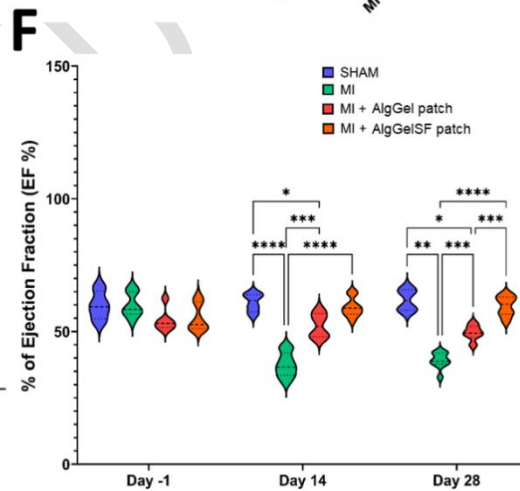
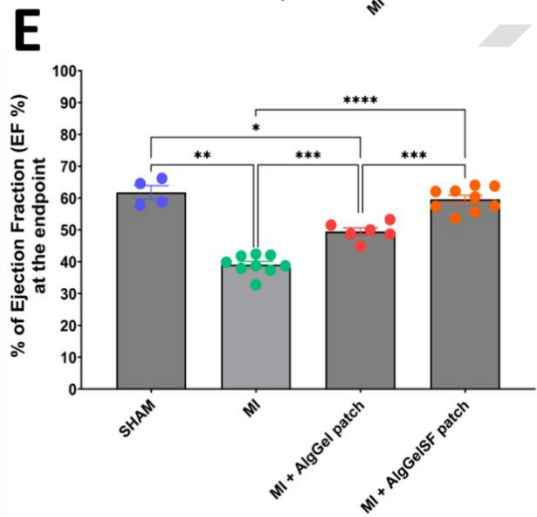
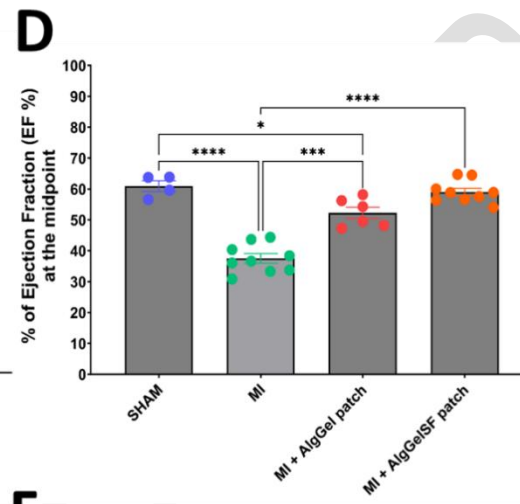
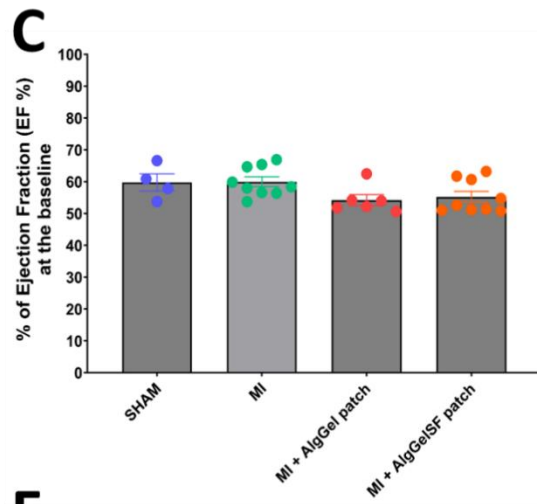
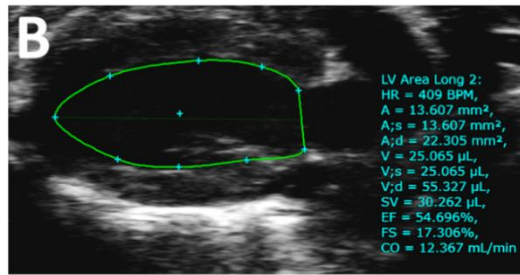
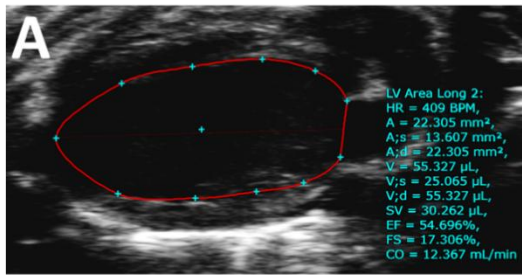


Figure 4. AlgGelSF-containing patches can improve the left ventricular ejection fraction % of murine hearts post-MI.

(A-B) The images are representative of a murine parasternal long axis US data analysis using VevoLAB software. The US data correspond to the US cycles of a mouse with MI performed and with the addition of the SF-containing patch. For each US data, both diastole (A) and systole (B) cycles are outlined using either the manual or automatic (AutoLV) modes. For each cardiac cycle, the software also shows data relative to: heart rate (HR, bpm), area of the cycle (A, mm²), area of systole (A;s, mm²), area of diastole (A;d, mm²), volume of the cycle (V, µl), volume of systole (V;s, µl), volume of diastole (V;d, µl), stroke volume (SV, µl), left ventricular ejection fraction (LVEF, %), fractional shortening (FS, %), cardiac output (CO, ml/min).

(C-E) The plots show the LVEF% analyses of US data acquired on parasternal long-axis view through Vevo 3100 Preclinical Imaging System (FUJIFILM VisualSonics, Toronto, Canada). The LVEF% values were analyzed at the baseline (1 day before the surgery) (C), at the midpoint (after 14 days) (D) and at the endpoint (after 28 days) (E) US data were analyzed using the software Vevo LAB 5.7.1 software (FUJIFILM VisualSonics, Toronto, Canada) and the statistical performed is a Brown-Forsythe and Welch ANOVA test, together with a Dunnett's T3 multiple comparisons test. Data are represented as mean with standard error mean. (F) The figure is representative of the LVEF% analyses of all mice experimental groups in the specific 3-time points (baseline, day -1; midpoint, day 14; endpoint, day 28). The statistical analysis performed is a two-way ANOVA test with Tukey's multiple comparisons test and data are represented as median (n≥4, * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001) (US=ultrasound, MI=myocardial infarction, Alg=Alginate, Gel=Gelatin, SF=silk fibroin).

3.3. Myocardial infarction models did not lead to hypertrophic tissue formation

To evaluate whether there was an increase in hypertrophic tissue in mouse hearts after MI, heart weight values were compared to body weight and/or tibia length values. Assuming that myocardial remodeling leads to an increase in scar tissue in the myocardium wall, there should also be an increase in heart weight, while the length of the tibia remains unchanged.

The results from **Figures S4A** and **S4B** show no measurable hypertrophy compared to the Sham group (heart weight (mg) / body weight (mg) = mean values between 0.004 and 0.009; heart weight

(mg) /tibia length (mm) = mean values between 7 and 11 mg/mm), further supported by the absence of statistical significance across the results.

3.4. Silk fibroin-containing patches protect against fibrosis in the myocardial infarction model

To assess the amount of fibrotic tissue generated after MI, and to measure the accumulation of heart muscle fiber within the cardiac tissue post-MI, H&E, Masson's Trichrome and Picro-Sirius Red staining were performed. As shown in **Figure 5A** and **Figure S3A**, the pattern of cardiac muscle fibers in the Sham group shows straight lines, supporting their alignment for proper function. For the MI group (**Figure 5B**, **Figure S3B** and **S4B**) the muscle fibers are only partially aligned, while both cells and ECM are disorganized. The muscle fibers tend to align again in the presence of AlgGel patch (**Figure S4C** and **S5C**). The MI procedure followed by the transplantation of AlgGelSF patch (**Figure 5D**, **Figure S4D** and **S5D**) displayed a similar fiber alignment to the Sham group.

Masson's Trichrome staining (**Figure 5A-D**) was utilized to identify the connective tissue and evaluate fibrosis, visualizing the distribution of collagen fibers in murine cardiac tissues post-MI. Cardiac muscle fibers are stained in purple, whereas collagen fibers are stained in blue [31, 32]. **Figure 5E** shows the analysis of the percentage of fibrotic tissue present in the 4 experimental mice tissues, calculated as the percentage of the ratio between the total fibrotic tissue area and the total tissue area. The Sham group presents aligned collagen fibers (**Figure 5A**) and a minor area of fibrotic tissue ($3.99\% \pm 2.51$) (**Figure 5E**), corresponding to a percentage of collagen fibers area versus the whole tissue. The MI group (**Figure 5B**) displays a partial organization of the muscle fibers and a larger region of fibrotic tissue with a higher density of collagen fibers ($13.22\% \pm 7.05$), ~9% higher than the Sham group. The MI with AlgGel patch group (**Figure 5C**) (10.68 ± 6.79) showed a decrease in the fibrotic region, with the muscle fibers tending to be more aligned, even though the amount of fibrotic tissue analyzed was ~3% less compared to the MI group. The MI with the addition of AlgGelSF patch (**Figure 5D**) (5.42 ± 4.93), displays more aligned muscle fibers and a lower density of collagen fibers, ~5% less compared to MI with the AlgGel patch group and ~8% less than the MI group. The amount of fibrotic tissue in MI with AlgGelSF patch

corresponds to a percentage of fibrotic tissue area, which is similar to the Sham group with no significant difference.

Picro-Sirius Red staining (**Figures S5A-D**) was used as a complementary method to stain collagen fibers with cardiac muscle fibers stained in yellow, and collagen fibers stained in red ^[29, 30]. Cardiac muscle fibers are aligned and well-organized in Sham group (**Figure S5A**), whereas MI group (**Figure S5B**) presents a partial muscle fibers alignment and disorganization of the collagen fibers. The MI group also presented a higher density of collagen fibers, thus a larger fibrotic region. A decrease in the amount of fibrotic tissue in the MI with the addition of the AlgGel patch group (**Figure S5C**) was observed. Furthermore, the collagen fibers and muscle fibers tended to re-align. The MI with the addition of the AlgGelSF-containing patch group (**Figure S5D**) displayed both muscle and collagen fibers aligned and less fibrotic tissue deposition.

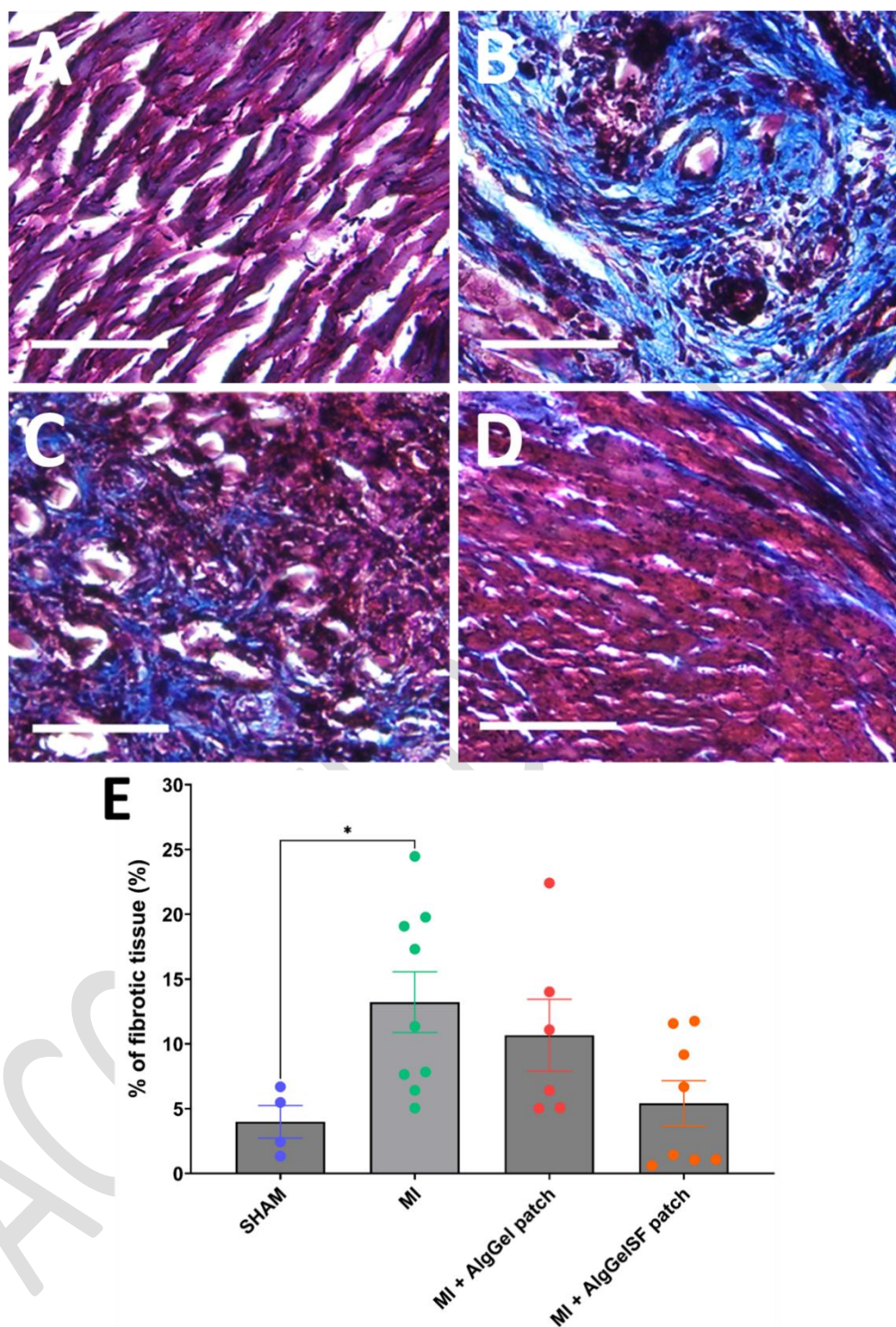


Figure 5. Masson's Trichrome staining of Sham, MI, MI with the addition of AlgGel patches, MI with the addition of AlgGelSF-containing patches and analysis of fibrotic tissue presence. (A-D) Representative images of Masson's Trichrome histological staining, acquired through Olympus BX61 Upright Fluorescence Microscope. Samples' images are related to the 4

experimental groups: Sham (**A**), MI (**B**), MI with the addition of AlgGel patch (**C**), and MI with the addition of AlgGelSF patch (**D**). The staining marks the collagen fibers in blue, while cardiac muscle fibers are stained in purple (magnification=20X, scale bar=100µm). (**E**) The plot is representative of the analysis performed on tissues stained with Masson's Trichrome histological staining, related to the 4 experimental groups. The plot shows an increase in the percentage of fibrotic tissue for mice with MI, followed by a progressive decrease of fibrotic tissue in mice with the AlgGel patches and mice with the patches containing AlgGelSF. The statistical analysis is a Brown-Forsythe and Welch ANOVA test, with a Dunnett's T3 multiple comparisons test. Data are represented as mean with standard error mean and the statistical analysis shows a significance between Sham and MI data (* $p < 0.05$, $n \geq 3$, MI=myocardial infarction, Alg=alginate, Gel=Gelatin, SF=silk fibroin).

3.5. Silk fibroin-containing patches support the expression of genes regulating cardiac remodeling in MI mice

Bulk RNA sequencing on murine models was performed to evaluate mouse transcriptomes and analyze the expression of specific gene sequences involved in cardiac remodeling after MI [37, 38]. RNA isolation and sequencing were performed for the following animal experimental groups: Sham, MI, MI with the addition of AlgGel patch and MI with the addition of AlgGelSF patch [17]. The principal component analysis (PCA) explains the variability in the gene expression of mouse transcriptomes [39]. The PCA plot (**Figure 6A**) represents the genome-wide expression profiles of the samples and is characterized by two Principal Components (PCs). At the same time, the first PC represents the primary difference in gene expression between the four experimental groups, showing a variance of 31.53%, the second PC represents a secondary variation in gene expression, due to individual sample variability, showing a variance of 15.16%. The first and second PC account for 46.69% of the total variance in the dataset. Sham samples show consistency in their gene expression profiles, whereas MI samples display a distinct expression profile due to the MI. **Figure 6A** reveals distinct clustering patterns, indicating a substantial gene expression difference across the four experimental groups: Cluster 1 represents the Sham (control) group, Cluster 2 represents MI group, Cluster 3 represents MI with the addition of AlgGel patches, and Cluster 4 represents MI with the addition of AlgGelSF patch group. Cluster 4 is well-separated from Cluster

2, suggesting that MI with the addition of AlgGelSF patch induces a significant shift in gene expression compared to the MI samples. Samples from Clusters 1 and 2 share similarities in their gene expression profiles.

The differential gene expression analysis identified 168 transcripts across the four experimental mice groups, relying on cut-off standards with fold-change (FC) of gene expression ratio > 1.5 and $FC < -1.5$, and p-value of 0.05. The analysis includes up- and down-regulation of the genes; a fold-change > 1.5 means upregulation, while a fold-change < -1.5 means downregulation ^[40]. The variations in gene expression led by the molecular responses pre- and post-MI are represented by the heatmaps in **Figures 6B-C** ^[41]. Our hierarchical clustering analysis of the murine transcripts shows clear gene expression patterns of upregulation (red) and downregulation (blue). **Figure 6B** shows the differential gene expression analysis between Sham, MI, MI treated with AlgGel patch and MI treated with AlgGelSF patch groups and reveals distinct transcriptional changes, which diverge between the four groups. **Figure 6C** directly compares more refined gene expression patterns between MI and MI treated with AlgGelSF patches. The Ingenuity Pathway Analysis (IPA, **Figures 6D-E**) comparing MI and MI treated with AlgGelSF patches highlights the enrichment of genes associated with specific post-MI pathways by the addition of AlgGelSF patches ^[42, 43]. **Table 1** indicates the top 64 transcripts that are differentially expressed in MI mice following the addition of AlgGelSF patches. These transcripts indicate an enrichment across specific categories, such as inflammatory response and disease, metabolic disease, lipid metabolism and redox signaling (**Table 1**). IPA also includes the up- and down-regulation of genes involved in cardiac tissue remodeling and fibrosis post-MI, such as COL4A5 and COL5A2, inflammatory response and cellular growth and proliferation, including BMI1, CD247, CD52, GNA12, NCOA4, PPARGC1A, TREM2, TXN, TYROBP. Genes like BMI1, CD247, COL4A5, COL5A2, GNA12, NCOA4, and PPARGC1A are upregulated, thus showing an increase in the amount of expressed transcript, while CD52, TREM2, TXN, and TYROBP are downregulated (**Table 2**).

Table 1. IPA analyses of genes differentially expressed associated with disease and molecular cell function pathways in MI with the addition of AlgGelSF patch compared to MI mice.		
Category	P-value	mRNAs
Inflammatory Response	2.45e-06-3.37e-02	ANXA9, ATP2B1, BMI1, CD247, CD300A, CD52, CKB, COG5, COL4A5, COL5A2, CTSZ, DSG2,

		FAM227B, FAU, FTL, GADD45B, GNA12, HBA2, Hbb-b2, IL27, ITPKB, KLHL24, mir-142 (includes others), MSMP, Mt1, NCOA4, NT5DC2, PLTP, PMEL, PPARGC1A, PSMB9, PUS10, RENBP, RMC1, S100A6, SH3BGRL3, SYT6, TGIF1, TMEM179, TMSB10, TOB1, TPM3, TREM2, TXN, TYROBP, USP9X
Metabolic Disease	1.13e-05-2.72e-02	C18orf32, CD247, CD300A, CD52, CKB, COG5, COL12A1, COL4A5, COL5A2, CSTB, CTSZ, DNAH11, DSG2, EIF2S3, FAM227B, FTL, GADD45B, Gm15350, GPLD1, HBA2, Hbb-b2, ITPKB, mir-142 (includes others), Mt1, NCOA4, NELFCD, PIGF, PLTP, PPARGC1A, PSMB9, PUS10, RMC1, S100A6, SF11, TGIF1, TMEM205, TMSB10, TPM3, TREM2, TXN, TYROBP
Inflammatory Disease	4.43e-05-3.01e-02	ANXA9, ATP2B1, BMI1, CD247, CD52, CKB, COG5, COL4A5, COL5A2, CTSZ, FAM227B, FAU, FTL, GADD45B, GNA12, HBA2, Hbb-b2, IL27, ITPKB, KLHL24, mir-142 (includes others), Mt1, NCOA4, NT5DC2, PLTP, PMEL, PPARGC1A, PSMB9, PUS10, RENBP, RMC1, S100A6, SH3BGRL3, STMN1, SYT6, TGIF1, TMEM179, TMSB10, TOB1, TPM3, TREM2, TXN, TYROBP
Cardiovascular Disease	7.21e-05-3.01e-02	BMI1, CD52, COL4A5, COL5A2, DNAH11, DSG2, FTL, GDF1, HBA2, Hbb-b2, Il3ra, KLHL24, NCOA4, PLTP, PPARGC1A, STMN1, TGIF1, TMEM205, TREM2, TXN, TYROBP, USP9X
Lipid Metabolism	1.13e-05-3.33e-02	GNA12, GPLD1, ITPKB, NCOA4, PLTP, PPARGC1A, STMN1, TMEM150A, TREM2, TYROBP
Small Molecule Biochemistry	1.13e-05-3.33e-02	ATP2B1, CKB, EIF2S3, FTL, GNA12, GPLD1, HBA2, Hbb-b2, ITPKB, Mt1, NCOA4, NT5C, PLTP,

		PPARGC1A, RENBP, STMN1, TMEM150A, TREM2, TXN, TYROBP
Cellular Growth and Proliferation	1.15e-04-3.37e-02	BMI1, CD247, CD300A, CD52, COL12A1, CTSZ, DSG2, FTL, GADD45B, GNA12, Hbb-b2, IL27, ITPKB, mir-142 (includes others), Mt1, NCOA4, PIGF, PMEL, PPARGC1A, RMI1, S100A6, STMN1, TGIF1, TOB1, TREM2, TXN, TYROBP, USP9X
Free Radical Scavenging	1.67e-03-3.33e-02	BMI1, CD52, CSTB, FTL, HBA2, Hbb-b2, PPARGC1A, S100A6, TXN, TYROBP
Post-Translational Modification	4.33e-03-2.48e-02	FTL, KLHL24, LRSAM1, TXN

Table 2. IPA analyses of up- and down-regulated genes involved in cardiac tissue remodeling and fibrosis post-MI in MI with the addition of AlgGelSF patch compared to MI mice.

Gene	log2FoldChange	P-value	Up or down-regulation
BMI1	1.575391487	0.00049071	upregulation
CD247	1.583121873	0.000581403	upregulation
CD52	-1.535272912	0.00090823	downregulation
CKB	-1.533103586	0.0000313	downregulation
COL4A5	1.60725235	0.001040459	upregulation
COL5A2	1.58796785	7.75E-09	upregulation
CTSZ	-1.631591967	0.000016	downregulation
DSG2	1.522915252	0.001580242	upregulation
FTL1	-1.566612213	0.00000159	downregulation
GADD45B	-2.020518781	0.00000502	downregulation
GNA12	2.047700969	0.00000111	upregulation
HBA-A2	-1.716351681	0.00000269	downregulation
HBB-B2	-1.609325968	0.0000134	downregulation

ITPKB	1.619015918	0.000767415	upregulation
KLHL24	1.964787241	0.00000112	upregulation
MIR142B	-1.58076208	0.003309117	downregulation
MT1	-1.671998712	0.000000333	downregulation
NCOA4	1.72066851	0.000245836	upregulation
PLTP	-1.687433089	0.00000147	downregulation
PPARGC1A	1.706034054	0.000279861	upregulation
S100A6	-1.512530904	0.00000701	downregulation
STMN1	-1.637487265	0.000139962	downregulation
TGIF1	-1.500613526	0.005825072	downregulation
TREM2	-2.024632196	0.00000308	downregulation
TXN1	-1.553009438	0.0000859	downregulation
TYROBP	-1.522702904	0.000160021	downregulation

4. Discussion

This study describes the effects of 3D bioprinted cell-free AlgGelSF-containing patches transplantation into an MI murine *in vivo* model to identify the potential cardioprotective effects. Overall, this study demonstrated that these patches provide protection against the MI-induced reduction in LVEF% and fibrosis, as well as modulating genes which regulate important myocardial disease mechanisms.

The transplantation of 3D printed patches containing AlgGelSF in an MI mouse model yielded promising results, showing the effectiveness of the surgical procedure methodology. This was possible due to the tensile strength (**Figures S1E** and **S1F**) and elasticity features exhibited by AlgGelSF-containing materials, which provide greater resistance to deformations and improve handling abilities (**Figures S1A-D**). The tensile strength testing also revealed the exceptional stretching features of AlgGelSF-containing patches, demonstrating SF's ability to adapt and resist to any deformations during cardiac tissue contraction (**Figure S1F**) [2, 9, 19].

The elastic properties of SF could allow the 3D patch to adjust and better adapt to the irregularities of the cardiac tissue micro-environment, without losing its structural integrity [44-46]. Our qualitative analyses support an improvement in handling abilities with the AlgGelSF-containing

patches, which also contributed to an enhanced surgical precision in positioning the patch, compared to the AlgGel r patch formulation (**Videos S5** and **S6**, respectively).

We previously demonstrated that increased concentrations of SF in AlgGel formulations improved the structural and mechanical properties of AlgGel hydrogels, as well as improving durability in physiological environments, respectively ^[9]. This is due to the presence of β -sheets in crosslinked 3D printed patches, which enhance and strengthen the hydrogen bonding within the SF structure, and delay the degradation process of AlgGel hydrogels, which tend to degrade faster in physiological environments ^[9]. The improved mechanical stability of the AlgGelSF-containing patches, possibly due to the strengthened hydrogen bonding and electrostatic interactions ^[9], while the presence of RGD sequences (from gelatin) promotes the cell surface integrin receptors, which may additionally support adhesion of the patch to the heart ^[47-50].

The absence of hypertrophic tissue following the transplantation of both AlgGel and AlgGelSF patches (**Figure S6**) was an interesting finding, as hypertrophy can lead to heart failure and further pathological complications ^[51]. The ability of the transplanted AlgGelSF-containing patches to prevent cardiac tissue hypertrophy suggests a possible positive interaction between the patch and the native cardiac tissue, which could be further explored in future work.

To evaluate the cardiac function of all the experimental groups and whether there was an improvement in MI mice that received the treatment with the SF patch, US data were analyzed for the LVEF% at specific time points, as shown in **Figure 4C-F**. A positive result was the efficacy of the 3D printed patches containing AlgGelSF on cardiac function. The LVEF% seen in MI mice that received the treatment with AlgGelSF patches, was improved by ~10%, compared to the MI mice that received the AlgGel patches, and by ~20%, compared to MI mice, after 28 days from the treatment. This result was not significantly different from the healthy mice group (Sham), demonstrating an improvement in cardiac function post-MI ^[52, 53].

The analysis of cardiac function was supported by histological analyses to evaluate the amount of collagen fibers (expressed as a percentage of fibrotic tissue area) that deposit after MI. MI mice treated with the AlgGelSF patch displayed lower amounts (in percentage) of fibrotic tissue in the infarcted area, compared to MI mice, with the mice treated with AlgGel patches, showing similar results to the Sham group. These results support a protective role of the AlgGelSF-containing patch against cardiac fibrosis ^[44, 52].

The improvement in cardiac function was also supported by the transcriptomic results. Our PCA analyses of the mice genome-wide expression profiles (**Figure 6A**) revealed a distinct cluster formed by the MI group, indicating a profound transcriptomic shift due to the ischemic injury. This is correlated with the US analysis after 28 days, where there was a decrease in LVEF% of ~23% compared to the Sham group (**Figures 4E and 4F**). Interestingly, MI with the addition of AlgGelSF patch group clustered further away from both MI and Sham groups, suggesting potential additional mechanisms regulating the increase in LVEF% following the addition of AlgGelSF. In addition, gene expression results shown in **Figure 6C** suggest that AlgGelSF patch might attenuate inflammatory and fibrotic responses while promoting pathways involved in cardiac tissue repair and prevention of excessive collagen fibers during cardiac muscle tissue remodeling ^[5, 52].

We used degummed silk fibroin as an inert material, so to avoid any potential immune response due to the material used. This is critical to prevent any potential complications in humans, supporting its long-term safety for cardiac applications ^[54]. However, the enrichment analysis for genes differentially expressed (**Figure 6D-E and Table 2**) supported the role played by AlgGelSF patch in modulating the inflammatory response, as well as the loss of cardiomyocytes through the downregulation of CD52, TYROBP, STMN1 and GADD45B genes ^[55-59]. The enrichment analysis supports SF's protective effects through the upregulation of genes like BMI1, PPARGC1A, DSG2 and KLHL24, shown to regulate cardiac tissue regeneration, lipid metabolism and structural integrity, respectively ^[60-63]. Of note, the increase in expression of CD247 and CD52 (**Tables 1 and 2**), both expressed in Natural Killer (NK) and T cells, supports a role played by NK cells in the absence of T cells in Rag1 mice. These genes were differentially expressed only in MI plus AlgGelSF, but not in MI plus AlgGel, as confirmed by **Tables S1 and S2**. This would indicate a role for the first-line defense, which has been shown to play a protective role against cardiac fibrosis ^[64]. These transcriptomic results are consistent with our histological results and support an inflammatory-mediated protection against cardiac fibrosis and reduction in LVEF%.

However, our study presents a few limitations. Future work should focus on formulating AlgGelSF-containing bioinks incorporating cardiac spheroids composed of cardiomyocytes, endothelial cells, and cardiac fibroblasts. These spheroids are expected to proliferate and fuse after printing, better recapitulating the native 3D cardiac microenvironment and enhancing functional integration with the host myocardium ^[17, 65].

While the acellular AlgGelSF-containing patch enables assessment of biocompatibility and mechanical support of the infarcted tissue, the absence of cells limits its capacity to restore active myocardial function. Therefore, evaluating cell-laden patches is a critical next step to promote contractility, vascularization, and electromechanical coupling. In addition, although murine MI models are essential for initial *in vivo* evaluation, their anatomical and physiological differences from the human heart restrict direct clinical extrapolation. Therefore, the effects of the use of AlgGelSF-containing patches in immune competent animals should be evaluated to improve clinical translatability to humans. Further analyses of the patch retention, degradation, or integration with host tissue during the 28-day implantation period will be critical to better understand the performance of the hydrogel *in vivo*. Additionally, other concentrations of SF could be tested *in vivo* as well. Of note, infarct size was not quantified to correlate functional recovery with structural cardiac repair. We also acknowledge that independent validation *via* either qPCR or immunohistochemical staining analyses will further validate our transcriptomic analyses and results. Our tensile testing was limited to qualitative observations, while quantitative measurements, such as tensile strength, elastic modulus, or elongation at break will be critical to evaluate the performance of the printed hydrogel under dynamic conditions. Progression to large-animal models, such as pigs, will allow the use of clinically relevant patch sizes and surgical techniques, better reflecting human cardiac anatomy and biomechanics. Successful validation in these models will be pivotal for advancing 3D bioprinted AlgGelSF-based cardiac patches toward clinical application.

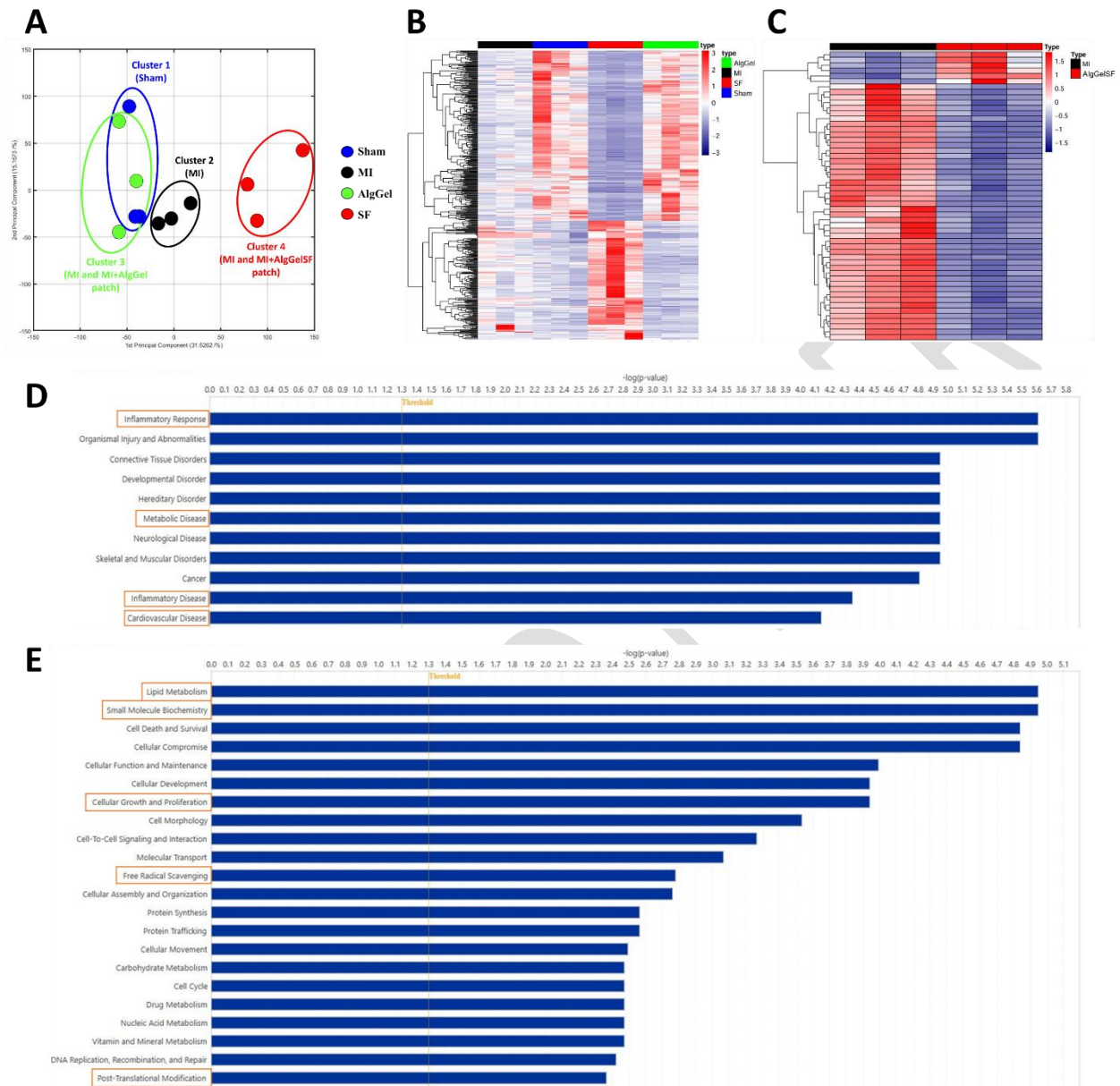


Figure 6. Transcriptomic profile of MI mice treated with AlgGelSF patches. (A-E) Representative images derived from RNA sequencing analysis performed on experimental groups: Sham, MI, MI with the addition of AlgGel patch and MI with the addition of AlgGelSF patch. **(A)** The image represents the principal component analysis (PCA) of the whole transcriptomic profile. **(B-C)** The two heatmaps show the gene expression levels of differential genes expressed between: i) Sham, ii) MI, iii) between MI and MI with the addition of AlgGel patch **(B)** and iv) between MI and MI with the addition of AlgGelSF patches **(C)**. **(D-E)** Representative images of the enrichment analysis for genes differentially expressed between MI and MI with the addition of AlgGelSF patch

experimental groups: diseases (**D**) and molecular cell functions (**F**) ($n \geq 3$, Fold Change of > 1.5 and < -1.5 , p -value=0.05, MI=myocardial infarction, Alg=alginate, Gel=Gelatin, SF=silk fibroin).

5. Conclusion

The 3D printed AlgGelSF-containing patches protect against the reduction in cardiac function post-MI by improving the LVEF%, reducing cardiac fibrosis, and by modulating the expression of genes regulating the immune response. Altogether, these findings support the potential use of AlgGelSF-containing hydrogels as a promising approach for cardiac protection in *in vivo* applications.

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Conflict of interest

The authors declare they have no competing interests.

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Ethics approval and consent to participate

All procedures were approved by the Animal Ethics Committee at the University of Technology Sydney (ETH19-4338).

Consent for publication

Not applicable.

Availability of data

Data are available from the corresponding author upon reasonable request.

Further disclosure

Part of or the entire set of findings have been presented in a conference, academic seminars and meetings.

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