Assessment of Electromechanically Stimulated Bone Marrow Stem Cells Seeded Acellular Cardiac Patch in a Rat Myocardial Infarct Model

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ABSTRACT

Although substantial progress has been made in tissue engineered cardiac patch, the translation of such strategies into a clinical setting has always face setbacks due to the lack of appropriate scaffolds and cell resources. Here, we evaluated cardiomyogenic differentiation of electromechanically stimulated rat bone marrow-derived stem cell (rt-BMSCs) on an acellular bovine pericardium (aBP) as well as the performance of this engineered patch in a rat myocardial infarct (MI) model. Briefly, aBP was prepared using a detergent-based decellularization procedure. The formed aBPs were seeded with rt-BMSCs and then stimulated under electrical, mechanical or electromechanical conditions (3-millisecond pulses of 5 V/cm at 1 Hz, 5% streching) to enhance cardiomyogenic differentiation. Thereafter, electromechanically stimulated patch was applied on MI region over a time period (3 weeks). After this period, the retrieved patch and infarct region were evaluated in terms of calcification, inflammatory reaction (CD68), cell migration from patch to host tissue and sarcomere structure related protein expressions. Patch related calcification was not examined in all tested group. Moreover, higher number of BrdU-labelled cells and

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low level of CD68 positive cells were observed in the infract region under electromechanically stimulated conditions as compared with static conditions. More importantly, MHC, SAC, Troponin T and N-cad positive cells were observed in both infarct region and retrieved engineered patch after the 3 weeks. As a result, we showed that a noticeable differentiation of stem cells on an acellular patch into cardiomyocytes under the electromechanical stimulation. This patch successfully integrated with the host tissue via cell migration from the patch to the infarct region.

Key words: Cardiac tissue engineering, rt-BMSCs, acellular bovine pericardium, electromechanical stimulation, myocardial infarct model.

INTRODUCTION

Myocardial infarction (MI) is an ischemic event that causes cell death and inflammation in myocardium (Alcon et al., 2012). Following MI, scar tissue arises due to migration of fibroblast and endothelial cells (van den Borne et al., 2010) and this results in a formation of a thin myocardial wall which causes heart failure and death (H. Wang et al., 2010). Hence, development of new treatment strategies is still very essential for the unmet clinical demand in treating MI (Karpov et al., 2017). Mesenchymal Stem Cell (MSC) therapy is one of the promising treatment strategies for MI, however it has substantial limitations such as low retention/engraftment rate (only 0.1% to 10% of injected cells), poor survival rate, off-target migration and short storage life (Tang et al., 2018; Zhang et al., 2018). Although it shows positive results in terms of feasibility and safety of stem cell delivery to the heart, significant functional benefit of stem cell therapy is yet to be achieved (Steele et al., 2017). Moreover, it is reported that implanted MSCs infrequently differentiate into cardiomyocyte at MI region (Gnecchi et al., 2013). It has also been mention that the rarely observed success of MSCs therapy could be as a result of secreted soluble factor as supposed to cardiomyogenic differentiation of stem cells (Mirotsou et al., 2011). Stem cell-derived cardiomyocytes were shown to be more efficient than undifferentiated MSCs in terms of myocardial regeneration. In a study, it was demonstrated that MSC-derived cardiac-like muscle cells improved functional myocardial generation more efficiently (T.-S. Li et al., 2005; Tomita et al., 1999). More often, the cardiomyogenic differentiation of MSCs *in vitro* is carried out using 5-Azacytidine (5-aza) (Joshi et al., 2018) or combination of 5-aza and TGF-β1 (Shi et al., 2016), yet the efficiency is low.

Therefore, it is crucial to safely deliver stem cells by using a scaffold to increase long-term treatment efficacy and to provide suitable microenvironment for survival and differentiation on the myocardial defect site.

Myocardial tissue engineering is an approach involving the use of appropriate cells and materials to mimic an *in vivo* myocardial structure. Myocardial tissue engineering is evolving more towards biological approaches, and thus strategies based on biophysical stimuli are gaining momentum. In this regard, acellular cardiac patch as scaffold has received a lot attention in tissue engineering. The main reason behind this popularity is that it supports cell behaviors in terms of retention, migration, proliferation and differentiation by preserving vital components of ECM such as functional and structural proteins, glycosaminoglycans (GAGs), glycoproteins, and bioactive factors (Bourgine et al., 2013; DeQuach et al., 2011; Yang et al., 2010). Also, it was reported that using a cardiac patch results in a 10-fold higher engraftment rate as compared with direct myocardial injection of cells (Gao et al., 2018; Nguyen et al., 2016). There are numerous clinically approved bovine-based patch products in the market. For instance, CardioCel and PhotoFix® are decellularized bovine patch that are currently available in the market (Iop et al., 2018). On the other hand, heart function is based on excitation-contraction coupling and mechanical contraction; cardiac cells are under the influence of both electrical and mechanical forces which regulate gene expression and cellular function. Therefore, it was hypothesized that mimicking the biophysical environment of the heart could increase the cardiomyogenic differentiation of MSCs. In several studies, researchers have applied electrical (Genovese et al., 2009; He et al., 2019), mechanical (Y. Huang et al., 2012; Shradhanjali et al., 2015) and electromechanical stimulation (Llucià-Valldeperas et al., 2017; B. Wang et al., 2013; Yoon et al., 2017) to MSCs for induction of cardiomyogenic differentiation.

In this study, we proposed a novel strategy which involves the use aBP as a scaffold for cardiomyogenic differentiation of rt-BMSCs under electromechanical stimulation. We evaluated the performance of electromechanically stimulated rt-BMSCs seeded on acellular bovine scaffolds using an experimental rat MI model. The first step involves decellularization in order to obtain a decellularized bovine pericardium matrix with preserved collagen and elastic fibers using a combination of physical and chemical treatments. Then, acetic acid, enzyme, and genipin treatments is carried out to prepare the

appropriate material for cell culture and *in vivo* experiments. Thereafter, the rt-BMSCs is engrafted into the aBP and following incubation with 10 μM 5-aza for 24 h, electromechanical stimulation is applied in order to induce cardiomyogenic differentiation. Finally, this electromechanically stimulated recellularized aBP is tested to define calcification, inflammatory reaction (CD68), cell migration from patch to host tissue and sarcomere structure related protein expressions using a rat MI model.

MATERIAL AND METHODS

Decellularization of Bovine Pericardium

Fresh bovine pericardium was procured from pericardial sac on ventral surface (Mendoza-Novelo et al., 2011) of the heart of calves aged 24 months and kept in physiological saline solution. Samples were cut into pieces of 2 cm x 2 cm and rinsed with PBS. Following the three cycles of freeze-thaw in PBS(Bai et al., 2014), samples were incubated with 0.35 mg/L(Chang et al., 2002) PMSF, (Biomatic, USA) contained hypotonic tris buffer (10 mM, ph 8.0) at 4°C during 24 or 48 h. Samples were then immersed in 1% Triton-X100 (Sigma Aldrich, USA), 0.5% sodium dodecyl sulfate (SDS, Sigma Aldrich, USA) and cocktail of both for 24 h, at hypotonic tris buffer (Courtman et al., 1994) at 4°C with constant stirring. Following rinsing with PBS for 48 h, pericardium was digested with 0.2 mg/mL DNAse (Biomatic, USA) and 150 mg/mL RNAse (Biomatic, USA) solutions at 37°C for 1 h to eliminate the antigenic factors (Chang et al., 2002). Hematoxylin and Eosin (H&E), Masson's Trichrome (MT), and Verhoeff van Gieson staining were performed to evaluate the decellularization procedure as explained in the supplementary data. Subsequently, procedures for pore adjustment, fixation and cytotoxicity were carried out (Supp. Data).

Stem Cell Viability and Morphology on Acellular Bovine Pericardium at Static Condition

rt-BMSCs were firstly isolated and characterized in terms of multi-lineage differentiation and expression of immunophenotypic antigens (details given in supplementary data). Then, to evaluate cell viability, these cells were seeded ($1x10^6$ cell/mL) onto acetic acid-conditioned and collagenase-treated aBP. After 7 days, the cell viability and cell morphology were determined using Live/Dead® viability/cytotoxicity kit and SEM, respectively (details given in supplementary data).

Electromechanical Stimulation of Acellular Bovine Pericardium Seeded with Stem Cells

Biomimetic Bioreactor System

To mimic the biophysical environment of the heart, we used a biomimetic bioreactor system (Ebers, TC-3 bioreactor with electrical stimulation module, Spain) to stimulate aBP seeded with rt-BMSCs. This system provides both electrical and mechanical stimulations, which imitate the physiological conditions of the heart. The mechanical stimulation is applied as uniaxial via a computer-controlled linear actuator, electrical stimulation on the other hand, is supplied using electrical stimulation module. Biocompatible teflon-coated platinum-iridium alloy (90-10) electrode wires are introduced into the culture chambers through septum connectors located in the chamber lid. To obtain synchronized mechanical and electrical stimulation, the system is controlled with a specific PC-based software. All the electrical and mechanical stimulation regimes used in this experiment were specified through the user interface of this PC-based software. Biomimetic bioreactor system was constructed in the cell culture hood after sterilization with autoclave and was used strictly following the manufacturer's instructions. The prepared aBP seeded with stem cells were properly placed into the chambers prior to physical stimulations (Fig. 1 A-C).

rt-BMSCs Differentiation under Electrical or Mechanical Stimulation

Here, we aimed at evaluating the individual effects of electrical and mechanical stimulations on rt-BMSCs differentiation into cardiomyocyte-like cells as well as the effects on the cell viability at different uniaxial stretching (5, 10 and 20% stretch of original scaffold length, frequency: 1 Hz, waveform: sinusoidal) (Y. Huang et al., 2012) and different electrical stimulation values (amplitude: 1, 3 and 5 V/cm, frequency: 1 Hz, pulse duration: 3-millisecond; waveform: square). To do this, rt-BMSCs were seeded onto aBP and cultured under static condition for 6 days to grow of cells and then after cardiomyogenic cell fate was induced chemically by using 10 µM 5-aza (details given in supplementary data). Stem cell seeded patch that chemically induced were mounted with grids into the chamber (n=3) and electrical or mechanical stimulations were applied. After stimulation for a period of 7 days, cell viability and gene expressions were investigated with Live/Dead® viability/cytotoxicity kit and qPCR, respectively. Unstimulated 5-aza-treated cells seeded aBP was used as control group.

rt-BMSCs Differentiation under Electromechanical Stimulation

The effect of electromechanical stimulation on rt-BMSCs' differentiation into cardiomyocyte-like cells was investigated using electromechanical stimulation regimes that are physiologically relevant (Fig 1D).

In this experiment, stimulation parameters were set based on parameters that gave optimum cell viability and qPCR results in experiments where electrical and mechanical stimulations were carried out separately. Electromechanical stimulation (amplitude: 5 V/cm, pulse duration: 3-ms, stretching: 5%, frequency: 1 Hz) was applied to cells seeded aBP (n=3) for 2 or 4 days. Before electromechanical stimulation, cardiomyogenic cell fate was induced with 5-aza. After 2 and 4 days, cardiomyogenic differentiation was investigated using qPCR. Unstimulated 5-aza-treated cell seeded aBPs were used as control group.

***in vivo* Assessment of Electromechanically Stimulated Stem Cells Seeded aBP Patch**

Preparation of BrdU labeled rt-BMSCs Seeded aBP Patch under Electromechanical Stimulation

For the identification of rt-BMSCs derived cardiomyocyte-like cells in host tissue, rt-BMSCs were labeled with BrdU (5-bromo-2-deoxyuridine), which is an analog of nucleoside thymidine. To achieve this, rt-BMSCs were exposed to BrdU Labeling Reagent (Invitrogen, USA) for 24 hours at 37°C (Tomita et al., 1999), and then the cells were detached from plate and seeded on acetic acid-treated and genipin-fixed aBP. After 6 days in static conditions, patches were exposed to cell culture medium containing 10 µM 5-Aza for 24 h in order to induce cardiomyocyte differentiation. Then, the patches were mounted into the chamber and electromechanical stimulation (amplitude: 5 V/cm, pulse duration: 3 ms, stretching: 5%, frequency: 1 Hz) was applied for period of 7 days. Finally, the stimulated patches were cut into pieces (0.5 cm x 0.5 cm and 800 µm thick) and implanted on MI region as explained below.

Transplantation of an Engineered Patch in a MI Rat Infarct Model

This animal study was performed in accordance with Kırıkkale University Animal Experiments Local Ethics Committee and all protocols were approved by committee (13/16 decision number). 18 adult Sprague-Dawley rats (350-500 g weight) were divided into three groups; Group-I: treated with electromechanically stimulated stem cells seeded aBP, Group-II: treated with unstimulated stem cell seeded aBP, and Group-III: treated with aBP without rt-BMSCs (control group). The experimental MI model in these rats was performed using cryoinjury method. Briefly, general anesthesia was induced using solutions like Xylazine and Ketamine, and thoracotomy was carried out. Afterwards, MI was induced by applying a cooled metal probe two times to the left ventricle free wall (10 s each time). Depending on the experimental group, electromechanically stimulated, unstimulated and null aBP were

grafted onto the injury area with fibrin glue (Fig 1 E-F). After 3 weeks, all animals were euthanized by a thiopental sodium (Pental 1 g, İ.E Ulagay, İstanbul) overdose. The tissues were collected for further analysis as described in supplementary data.

Statistical Analysis

The normality of MTT data was checked using Box and Whisker graph, and it was revealed that the data did not show Gaussian distribution. Thus, the cell viability results were analyzed using non-parametric Mann Whitney U-test (using GraphPad Prism 6, version 6.01). On the other hand, qPCR results were analyzed using two-way ANOVA (for electrical or mechanical stimulation experiments) and one-way ANOVA test (for electromechanical stimulation experiment), and statistical significance between groups was determined with Tukey test. The MTT experiment was conducted using 4 replicates, whiles all other experiments were conducted using 3 replicates. Data were shown as means ± SD and considered significant if $p<0.05$.

RESULTS

Decellularization of Bovine Pericardium

Histological results showed that all cell nuclei from bovine pericardium were successfully removed after hypotonic tris buffer treatment for 24 h and 48 h. Moreover, bovine pericardium was completely cell-free after treatment with 1% Triton-X100 and SDS/Triton-X100, and the wavy-like structure of collagen fibers was the same as for the native pericardium (Fig 2B-D). Contrary to this finding, collagen wavy-like structure was not clearly seen in group treated with SDS (Fig. 2C). Meanwhile, the elastic fibers observed were thicker and more visible in all the other groups than in native pericardium. In SDS group, shorter and fragmented elastic fibers were observed as compared to the other groups and also native pericardium (Fig 2C). Based on these results, we decided to use 1% Triton-X100 for further experiments (Fig 3B).

Pore Size Optimization, Fixation and Cytotoxicity of Acellular Bovine Pericardium

Image analysis indicated that pore size of aBP was 14.8±6.9 μm (Fig. 3C-I) and it was increased to 50.97±17.73 μm after 1 M acetic acid-0.5 U/mL collagenase treatment (Fig. 3C-II). Results from histological staining has revealed this treatment did not affect collagen wavy-like structure, however the elastic fibers were aggregated (Fig. 3C-III B and 3C-IV). Ninhydrin assay results has demonstrated that

acetic acid-treated aBP had a higher fixation index as compared to native pericardium (control group) at 0.05% genipin concentration, yet slightly lower at 0.5%. aBP fixation index was 37.9% at 0.05% genipin concentration and 51.1% at 0.5% genipin concentration. On the other hand, fixation index of native pericardium was 1.3% and 55.7% at 0.05% and 0.5% genipin concentrations, respectively. Cytotoxicity test results indicated that, acetic acid or genipin fixation treatments did not affect cellular viability, and even though it was significantly higher ($p<0.05$) than positive control, there was no significant difference between native pericardium and treated groups ($p>0.05$) (Fig 3D).

Stem Cell Viability and Morphology on Acellular Bovine Pericardium at Static Condition

Flow-cytometry and multi-lineage differentiation analyses were indicated that, isolated cells showed stem cell features. They differentiated into both adipogenic and osteogenic lineage and they highly expressed cell surface markers (CD29 and CD90) that are the indicators of stem cells (Dominici et al., 2006) (Fig. S1). According to Live&Dead staining results, rt-BMSCs could maintain their viability on acetic acid and collagenase-treated aBP even after 7 days (Fig 4A). SEM results indicated that, rt-BMSCs have elongated shape and are attached onto the surface of aBP (Fig 4B).

Effect of Different Stretching and Amplitude on Viability and Cardiogenic Differentiation of rt-BMSCs.

In order to investigate the optimum stretching and amplitude values, rt-BMSCs seeded aBPs were stimulated under electrical (1, 3 or 5 V/cm, 1 Hz) and mechanical (5, 10 or 20% stretching) conditions to enhance cardiomyogenic differentiation using a biomimetic bioreactor. The cell viability from Live&Dead staining experiment was found to be high for both mechanical and electrical stimulations after 7 days at all stretching and amplitude values, even though it varied between applied stretching and amplitudes parameters. For mechanically stimulated patch, negligible number of dead cells were found in groups with both 5% and 10% stretching, on the contrast, the dead cells in group with 20% stretching are more obvious (Fig 5 Panel A). In electrical stimulation experiment, small number of dead cells were seen in all groups, but the lowest number of dead cells were observed in 5 V/cm group (Fig.5 Panel B). It was also realized that, rt-BMSCs spread randomly at 5% stretching, but when the stretching value was

increased to 10 or 20%, the cells were aligned. Meanwhile, at each amplitude applied in the electrical stimulation experiment, cellular alignment was not observed.

qPCR results based on various stretching and amplitude values are shown in Fig 5. In mechanical stimulation experiment, 5% stretching resulted in statistically higher expression of NKX2.5 among all genes (3.5-fold) while other stretching parameters showed higher expression for GATA4 among all genes (3.8-fold and 2.6-fold for 10 and 20% groups, respectively) ($p<0.05$). In comparison with the control group, all targeted genes measured were higher in groups with 5% stretching (1.2, 1.5 and 1-fold for GATA4, MEF2C and CACNA1C, respectively). The NKX2.5 and CACNA1C detected were lower in groups with both 10 and 20% stretching (Fig. 5 Panel A). In the case of electrical stimulation experiment, MEF2C expression was computed to be significantly higher than NKX2.5, GATA4 and CACNA1C expression in both 1 and 3 V/cm group ($p<0.05$), and higher than GATA4 and CACNA1C in 5 V/cm group ($p<0.05$). GATA4 and NKX2.5 expressions were found to be higher in 5 V/cm group than in both 1 and 3 V/cm groups. As for CACNA1C expression, it was observed in 3V/cm and 5 V/cm group but not at 1 V/cm (Fig 5 Panel B). All taken together, we decided to continue with 5 V/cm as amplitude and %5 as stretching for electromechanical stimulation experiment

rt-BMSCs Differentiation under Electromechanical Stimulation

rt-BMSCs seeded acellular patches were stimulated electromechanically for two time periods and cardiomyogenic differentiation evaluated via qPCR analysis (Fig.6). At day 2, all targeted genes were higher in electrical stimulation group than mechanical and electromechanical groups (Fig 6A). GATA4, MEF2C and NKX2.5 expressions were 1.4 ($p>0.05$), 3 and 2.9-fold($p<0.05$) higher in electrical rather than electromechanical stimulation group, respectively. On the other hand, GATA4, MEF2C and NKX2.5 expressions were measured 2.9 ($p<0.05$), 1.33 ($p>0.05$) and 2.2-fold ($p>0.05$) higher in electrical stimulation group as compare to mechanical group, respectively. MEF2C expression was only significantly lower in electromechanical stimulation group than mechanical group. At day 4, expression of all targeted genes was significantly higher in electromechanical stimulation group($p<0.05$) (Fig 6B). Specifically, GATA4, MEF2C and NKX2.5 expressions were 2.5, 2 and 2-fold higher in electromechanical stimulation as compared to electrical stimulation group, respectively ($p<0.05$). In comparison with the mechanical stimulation, GATA4, MEF2C and NKX2.5 expressions were 2.3, 3.5

and 1.8-fold higher in electromechanical stimulation group ($p<0.05$), respectively. In accordance with all these results, we concluded that stimulation time can be a crucial parameter for cardiac differentiation of rt-BMSCs on aBP patch and electromechanical stimulation can be more effective than other stimulation at a long time.

***in vivo* Assessment of Electromechanically Stimulated Stem Cells Seeded Acellular Patch**

To assessment of electromechanically stimulated patch in terms of calcification, inflammatory reaction (CD68), cell migration from patch to host tissue and protein expressions that are in associated with sarcomere structure (MHC, SAC, Troponin T) and mechanical junctions (nCAD), we implanted electromechanically stimulated patch in a rat myocardial infarct model. Prior to *in vivo* experiment, stem cell seeded aBP patches were electromechanical stimulated during 7 days and then implanted in MI model. We used 7 days as stimulation duration, because our results suggested that longer stimulation time is more effective for differentiation of rt-BMSCs on aBP at applied parameters than short stimulation time. In addition, in a recently published work, researchers have used 7 days as stimulation time (Llucià-Valldeperas et al., 2017). After the 3 weeks on implantation, animals were sacrificed and histological and immunohistochemical staining were conducted for both infract region and retrieved patch.

Histological Staining Results

H&E staining demonstrated that MI was induced successfully in all groups (Figure 7). Whiles a fibrotic area consisting of spindle myofibroblast, fibroblast, capillary and collagenous tissues were seen at the defect site for electrostimulated and unstimulated groups, fibrotic tissues with hemorrhage as well as granulation with high content of blood cells and degenerated cardiomyocytes were observed in the control group. Von Kossa staining results showed that there was no calcium accumulation in all the groups. To identify the patch-derived cells in host tissue, BrdU staining was performed and the results presented in figure 7; we observed some BrdU-positive cells migrated out of the patch into the infarct region in both electromechanically stimulated and unstimulated group. Importantly, there were more BrdU-labeled cells in electromechanical stimulated group rather than static group. BrdU-positive cells were not observed in the control group as expected. We also examined BrdU positive and negative cells in retrieved patch clearly (Fig. S2).

Immunohistochemical Analysis Results

Immunohistochemical analysis results indicated that there were more MHC and Cardiac Troponin T positive cells in the infract region in groups under electromechanical stimulation than the unstimulated group. However, the expression of nCAD and SAC proteins was the same in both groups. Furthermore, there was lower CD68 cell expression in stimulated group as compared to unstimulated group. In the control group, it was the CD68 positive cells that were observed much more compared to other groups. More importantly, results showed that MHC, SAC, Cardiac Troponin T, nCAD and CD68 positive cells were present in the retrieved patch after 3 weeks (Fig. 8).

DISCUSSION

In this study, we investigated rt-BMSCs differentiation into cardiomyogenic lineage on aBP patch under biophysical stimulations and the performance of this patch on a myocardial defect in rat model. We used 1% Triton-X100, 0.5% SDS or cocktail of both in hypotonic tris buffer for incubation following freeze-thaw cycles for decellularization of bovine pericardium. In accordance with published data in the literature , all the applied procedures was successful in removal of cells, however collagen wave-like structure and elastic fibers of pericardium were damaged in groups treated with SDS (N. Li et al., 2018). Dong and co-workers treated acellular pericardium with 0.2 M acetic acid and reported the average pore size of the prepared pericardium was 162 ±24 μm(Dong et al., 2009). Interestingly, we obtained a much smaller pore size (51±17 μm). This difference in pore size could be attributed to differences in pericardium sources in terms of age or sex of bovine. Although, the pore size was smaller, rt-BMSCs on acellular matrix was very well attached with high viability (Fig.4). Aside this, we examined cells in the both upper and lower side of retrieved patch (Fig S2). Thus, we concluded that the pore size of our designed aBP could be appropriate for rt-BMSCs cultures. Moreover, MTT results showed no toxicity as a result of acetic acid treatment. Wei and colleagues treated aBP with acetic acid and collagenase, and presented in their finding that collagen distribution comparable with that of native tissue but no elastic fibers were observed (Wei et al., 2005). On the contrary, we clearly observed elastic fibers in both acetic acid/collagenase-treated and untreated group (Fig. 3). Patches need to be fixed by fixation reagents to decrease inflammatory reaction and calcification (L. L. Huang et al., 1998). Clinically, the most commonly used fixation agent is glutaraldehyde (H. W. Sung et al., 2003) yet this agent is toxic

(H.-W. Sung et al., 1999) and can cause calcification in long term (Golomb et al., 1987; Manji et al., 2006). For this reason, we used a natural cross-linking agent called genipin for fixation. Different genipin concentrations have been used in previous studies (Chang et al., 2007; Lim et al., 2012; Wei et al., 2005). For example, in some studies, researchers fixed biological tissues using a 0.625% aqueous genipin solution(H. W. Sung et al., 2001; Wei et al., 2005). In this study, we used 0.5% genipin solution and von Kossa staining results indicated that this genipin concentration was sufficient to suppress calcification but insufficient for total elimination of inflammatory reaction. So, we thought increasing the time of genipin fixation should help overcome these inflammation reactions. Cell viability assay results showed that the cell viability was comparable to the negative control after genipin fixation (Tsai et al., 2000). Based on the immunoreactivity results, the number of CD68 positive cells was high in control group and these cells were observed in retrieved patch as well. It is well-known that MSCs could modulate immune response (Molina et al., 2015), thus, the observed low level of CD68 positive cells in test group could be as a result of immunomodulatory effect of MSCs. On the other hand, as mentioned before we observed CD68 positive cells in retrieved patch and this result could be possibly attributed to the migration of macrophages from host tissue to patch.

It was reported that 5, 10, 15 and 20% strain applied to MSCs on elastic silicone membrane show no statistical difference in cell viability (Y. Huang et al., 2012) and a cellular alignment could be achieved if 10% stretching is applied to the stem cells (Y. Huang et al., 2012; Rabbani et al., 2016) or cardiomyocytes (Dhein et al., 2014) for 24 h. Another research group reported that, MSCs could detach from stretchable piezoelectric substrates or even die when more than 5% stretching is applied (Yoon et al., 2017). We also found that, dead cells become more significant at 20% stretching value than other tested values and more cells are aligned in both 10 and 20% group as compared to 5% group after 7 days (Y. Huang et al., 2012). Mechanical stimulation induced cardiomyogenic differentiation of MSCs on elastic silicone membrane (Amin et al., 2014; Bhang et al., 2010; Guo et al., 2011; Y. Huang et al., 2012) or stretchable piezoelectric substrates (Yoon et al., 2017). Our results showed that the level of progenitor (NKX2.5), early (GATA4 and MEF2C) and CACNA1C genes were increased and also the cardiomyogenic gene expression is dependent on the magnitude of stretching on aBP, just like it was reported for elastic silicon membrane (Y. Huang et al., 2012). For instance, while NKX2.5 expression

was higher in 5% stretching group, GATA4 expression was rather high at both 10 and 20% stretching ($p<0.05$). This is unlike what Huang and coworkers reported: GATA4, MEF2c and NKX2.5 expressions were higher in 10% stretching group. This difference could be possibly attributed to the used substrate for cell culture. Previous studies utilized a strain magnitude of 3% (Bhang et al., 2010; Yoon et al., 2017) or 10% (Y. Huang et al., 2012) for MSCs differentiation. In the present study, 10 and 20% stretching resulted in higher expression of only two genes (GATA4 and MEF2C), meanwhile 5% stretching resulted in higher expression of all targeted genes as compared to control group. Based on this, 5% stretching was selected as optimum parameter for electromechanical stimulation experiments.

It was observed that different amplitude values result in different level of mRNA expression of GATA4, MEF2C, NKX2.5 and CACNA1C (Fig 5 Panel B). We believe that amplitude-dependent cardiomyogenic differentiation could be due to differences in amount of ROS produced at tested amplitudes (Serena et al., 2009). Tandon and co-workers mentioned that 3 V/cm at 3 Hz is optimal for neonatal cardiomyocytes on collagen scaffold (Tandon et al., 2011). However, we used 5 V/cm amplitude in electromechanical stimulation experiments due to higher cell viability and MEF2C, GATA4 and NKX2.5 expressions. Also, our cell sources and scaffold material is different from the ones used by Tandon et al. The physiological values for electrical stimulation is known to be between 0.1-10 V/cm and for that matter, several studies used 5 V/cm amplitude (Au et al., 2007; Pavesi et al., 2015; Radisic et al., 2004). Only few groups have reported cardiomyocytes cell alignment under electrical stimulation (Au et al., 2007; Radisic et al., 2004). Much like other research groups, we also did not observe cellular alignment at all tested amplitudes (B. Wang et al., 2013). Since, the topography of surface is thought to crucial for the alignment of cells, we assume that the random nature of the distributed cell morphology might be due to the structure of natural disorganized collagen and elastic fibers of aBP (Cook et al., 2016; Llucià-Valldeperas et al., 2019). Also, the amount of live cells was higher in mechanical stimulation than in electrical stimulation group (Fig 6) similar to the findings in past studies, in which the cell number was found to be statistically higher in cardiomyocyte-encapsulated fibrin hydrogel stimulated with mechanical stretching (Morgan et al., 2014). Therefore, high viable cell number of mechanically stimulated aBP in this study might be because of the effect of mechanical stimulation on cell proliferation.

In this study, rt-BMSCs were seeded on stretchable aBP and exposed to constant electromechanical stimulation for 2 or 4 days to induce cardiomyogenic differentiation. We observed early cardiomyogenic gene expressions at day 2 and day 4 after electromechanical stimulation application. The level of targeted genes was higher in day 4, suggesting that longer stimulation time is more effective for differentiation of rt-BMSCs on aBP at applied parameters than short stimulation time. Wang and co-workers further reported that electromechanical stimulation does not only enhance cardiomyogenic differentiation of rt-BMSCs on acellular porcine myocardium, but also contractile protein expression (α-actinin, myosin heavy chain, and connexin-43) as compared to control groups (B. Wang et al., 2013). Wang et al., in their attempt to enhance cardiomyogenic differentiation, applied electrical and mechanical stimulations simultaneously, yet this does not properly mimic the normal physiological conditions of the heart (Morgan & Black III, 2014). We, however, utilized a biomimetic bioreactor system which creates a biophysical environment similar to that of the heart: in a cell culture chamber, electrical stimulation and stretching are applied to mimic excitation-contraction coupling mechanism of the heart (Fig 1D). Cardiac specific gene expressions for rt-BMSCs seeded on aBP was observed in this bioreactor conditions. Unlike in our case, Wang and co-workers used 20% stretching and 5 V amplitude at 1 Hz as electromechanical stimulation parameter for 2 days and detected cardiac related gene expressions.

In previous studies, cryoinjury was used to induce MI in mice or rats (Cho et al., 2006; Leferovich et al., 2001; Polizzotti et al., 2016; Ryu et al., 2005), however, due to technical difficulties of left anterior descending coronary artery (LAD) ligation, different results have been reported (Polizzotti et al., 2016). Nevertheless, our attempt to use cryoinjury to induce MI. Histology was successful indicating MI can be formed using this technique (Fig 7). After 3 weeks of *in vivo* implantation, BrdU-labeled cells were observed at both retrieved patch (Fig S2B) and in the infarct region (Fig 7). Aside this, CD68 positive cells were also observed in both retrieved patch and the infract region in all groups, which signifies the migration of cells from host tissue into implanted patch. One study has shown that migration of stimulated cells from fibrin patch to host tissue was only occasionally (Llucià-Valldeperas et al., 2017). However, we observed a relatively high number of migrated cells in the infract region and this might be due to stimulation of rt-BMSCs on a 3D aBP (made of collagen and elastic fibers) because cells are able

to sense their environment and the environmental factors tend to alter cell behaviors like growth and migration (Lu et al., 2019). Again, immunohistochemistry analysis revealed MHC, Cardiac Troponin T and SAC expressions in both retrieved patch and the infract region. These proteins are members of sarcomere structure which is the basic contractile unit of cardiac muscles(England et al., 2013). Hence, we thought that due to implantation of electromechanically stimulated stem cells seeded patch, contractile cardiomyocytes may arise in the infarct region. And, rt-BMSCs could differentiate into cardiomyocytes in the patch after the 3 weeks of implantation. It has been reported that biophysical stimulations upregulated the expression of connexin43 and N-cadherin which are responsible for electrical and mechanical junctions, respectively (B. Wang et al., 2013; Yamada et al., 2005; Zhuang et al., 2000). In our study, we observed nCAD positive cells in both retrieved patch and the infarct region of stimulation group but for connexin-43, we observed a very weak positive staining into retrieved patch (data not shown). This result encouraged our findings about arise of contractile cardiomyocytes in infarct region. Because, in cardiac tissue, adherens junctions connect neighbor cells through nCAD and enabling transmission of contractile force from one cell to another (Ferreira-Cornwell et al., 2002).

CONCLUSION

Mesenchymal stem cell based cardiac tissue engineering applications are essential to overcome challenges faced in stem cell therapy in treating heart-related problems like Myocardial Infraction. The biophysical microenvironment of the heart is one of the critical factors to be considered in order to design products with high therapeutic benefits. In this regard, we seeded rt-BMSCs on aBP and stimulated the cells using biomimetic bioreactor with electromechanical stimulation to induce cardiomyogenic differentiation. As a result, not only was the cardiomyogenic differentiation of rt-BMSCs enhanced, but also cells which expressed cardiac related proteins were seen in both infarct region and retrieved patch. Moreover, patch-derived stimulated cells were detected in the host tissues. These results suggest that electromechanical stimulation could be vital in enhancing pre-differentiation of MSCs on aBP-based patch, thereby creating a new window of opportunity for MI treatment.

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AUTHOR DISCLOSURE STATEMENT

All authors have stated explicitly that there are no commercial associations that might create a conflict of interest in connection with this article.

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Figure Legends

Figure 1. Representation of biomimetic bioreactor and its parts (A, B and C) and implantation of ready-to-use rt-BMSCs seeded aBP on MI region in rat MI model (E, F). A) Mechanical and electrical stimulation modules of biomimetic bioreactor system. B) Demonstration of how cell culture chambers were horizontally put onto motor unit of the reactor. C) Representation of how rt-BMSC seeded aBP is anchored into cell culture chamber via grids prior to stimulations. D) Graphical representation of electromechanical stimulation regimes. E) MI region created using cold metal probes. MI region (grey color) is indicated with yellow arrow. F) Implantation of electromechanically stimulated cardiac path (indicated with yellow arrow) on MI region with fibrin glue.

Figure 2. Verhoeff van Gieson staining results of A) Native pericardium, B) Pericardium treated with 1% Triton-X100, C) Pericardium treated with 0.5% SDS, and D) Pericardium treated with Cocktail of 1% Triton-X100 and 0.5% SDS in hypotonic tris buffer. Collagen fibers (brown) and elastic fibers (black) are indicated with red and yellow arrows, respectively. Scale bar: 50 µm.

Figure 3. A) H&E results of native pericardium. The native pericardium was used as a control group to examine the decellularization success in terms of removal of pericardial cells. Cell nuclei are stained with purple, while's collagen fiber with pink. B) Histological evaluation of bovine *pericardium* following treatment with 1% Triton-X100 in hypotonic tris-buffer for 48 h (B-I, BII, and B-III represent H&E, Verhoeff van Gieson and Masson Tricrome staining, respectively). SEM results after decellularization are shown in B-IV. SEM and Verhoeff van Gieson staining results of acellular pericardium (*C-I* and *C-III*, respectively) and acetic acid/collagenase-treated acellular matrix (C-*II* and *C-IV*, respectively). After Verhoeff van Gieson staining, collagen and elastic fibers were observed, under light microscopy to be brown and black colored, respectively. *D*) *Cytotoxicity* results of genipin (0.5% w/v) fixed and acetic acid (1M) treated *a*BP. Results are considered statistically significant if p value is smaller than 0.05 and this is shown with asterisk (*). nBP: Native Bovine Pericardium, aBP: Acellular Bovine Pericardium, AA: Acetic acid, Gen.: Genipin.

Figure 4. A) Cell viability of rt-BMSCs on aBP. B) rt-BMSCs morphologies on ABP. Red arrows show attached cells and blue arrows show fibrils structures of acellular matrix.

Figure 5. Effect of mechanical and electrical stimulations on rt-BMSCs viability and cardiogenic differentiation on aBP based on different stretchings and amplitudes. Panel A) Cell viability and cardiac specific gene expressions in mechanical stimulation experiment at different stretching values. Panel B) Effect of different amplitude values on stem cell viability and cardiomyogenic lineage differentiation in electrical stimulation experiment. In both experiments, cell viability was assessed with Live&Dead staining and cardiac specific gene expressions evaluated with qPCR. In Live&Dead staining, live and dead cells were observed in green and red colors, respectively. The analysis of qPCR result was performed using two way-ANOVA test and statistically significant differences between groups are indicated with asterisk (*) ($p<0.05$) or double asterisk (**) ($p<0.005$). Scale bar: 100 μm.

Figure 6. Cardiomyocyte related gene expressions after electrical, mechanical and electromechanical stimulations at A) day 2 and B) day 4. Statistical analysis was carried out using one-way ANOVA test and significant differences between groups were defined using Tukey post-hoc test. Cut off p value was set as 0.05 and results are considered significant if p is lower than 0.05 between groups as shown with asterisk (*). E. Sti: Electrical stimulation, M. Sti.: Mechanical stimulation, E.+M. Sti.: Electromechanical Stimulation.

Figure 7. H&E, BrdU and von Kossa staining of both electromechanically stimulated and unstimulated cell seeded aBP. Result showed successful MI formation and absence of any calcification on the implanted side after 3 weeks. Presence of BrdU-labeled cells in host tissue indicated that electromechanically stimulated stem cells could migrate from patch to host tissue after 3 weeks. BrdU positive cells are shown with yellow arrows. MI: Myocardial Infracted Region, N: Native Tissue Region.

Figure 8. Expression of cardiac specific protein and inflammation reaction marker on MI region after 3 weeks for both treated with electromechanically stimulated patch and treated with unstimulated patch. Retrieved patch was also examined for cardiac specific protein and inflammation reaction marker expression. Yellow arrows show protein expressions. MI: Myocardial Infracted Region, N: Native Tissue Region. Scale bar: 50 μm. SAC: Sarcomeric Alpha Actinin, MHC: Myosin Heavy Chain, nCAD: N Cadherin.