Research
Journal of Cardiology
RJC
OPEN ACCESS

Techniques of Fibroblasts and Cardiomyocytes Co-Cultures for Atrial Fibrillation Studies

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ABSTRACT

Background and Objective: Currently, heart diseases like cardiac arrhythmias are a high public health impact worldwide. One of the most frequent cardiac arrhythmias is atrial fibrillation (AF), a heart condition that causes an irregular and often abnormally fast heart rate. The AF had been linked with atrial fibrosis also, but the cause-effect relationship is not clear yet. This study aimed to simulate the conditions of interaction in atrial fibrosis, through different co-cultured proportions of NIH-3T3 rat fibrotic cells (fibroblasts) and HL-1 rat cardiomyocytes cells. **Materials and Methods:** Electrical activity in the cultures was then assessed by optical mapping to study the patterns of action potential propagation between cardiomyocytes, using the voltage-sensitive marker Di-8-ANEPPS. **Results:** The cultures were found to be distinguishable within the plates, according to dot and linear patterns performed by parafilm patterns. **Conclusion:** Cardiomyocyte's electrical activity could not be detected in co-cultures, which is necessary to establish the effect of the presence of fibroblasts, whereby studies should be performed with other conditions and measurements to establish possible changes in the patterns.

KEYWORDS

Fibrillation, fibrosis, arrhythmias, cardiomyocyte, fibroblasts, optical mapping, electrical activity

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INTRODUCTION

Cardiac arrhythmias are one of the major causes of morbidity and mortality worldwide[1] and one of them, perhaps the most frequent is atrial fibrillation (AF), whose incidence increases with age[2]. This consists of the presence of chaotic and desynchronized atrial activations or contractions and studies have found that its initiation and maintenance are due to several causes, like micro-fibrosis. In turn, the presence of micro-fibrosis in the atrial tissue is related to collagen accumulation in the extracellular space, which affects cellular coupling and thus the propagation of the electrical impulse between them[3-5]. For AF treatment, the most reliable method that exists and is currently applied is catheter ablation, which first measured the electrical activity inside the heart. Then, once the region where the arrhythmia problem originates is identified, the tissue is destroyed using thermal energy or very low temperatures. This creates small scars in certain parts of the cardiac muscle, which alters or eliminates the erratic electrical signals in the heart and cardiac rhythms are restored[6]. However, this method still requires better identification of the cells that form such tissues, since the procedure only establishes the trigger points of the arrhythmia, but not the specific composition of cells that could cause it. So it is necessary to better understand the mechanisms that accompany their formation and their relationship with the presence of atrial fibrillation[7].

ISSN: 2151-8297 (Online)
ISSN: 1819-3404 (Print)
https://doi.org/10.17311/rjc.2022.2.9

Received: 05 May 2021
Accepted: 09 Aug. 2021
Published: 01 Jan. 2022

The arrhythmogenic tissue formed in the atrium is largely a consequence of its structural remodelling[8,9], which occurs in a process known as atrial fibrosis. Here the composition of the atrial tissue is altered and as a consequence, its function[8]. *In vitro* and *in silico* studies have associated atrial fibrillation with the presence of certain fibroblast-like cells (myofibroblasts and fibrocytes) in the tissue, which couple ionically through channels with adjacent cardiomyocytes. The presence of these cells affects action potential propagation in the cardiomyocytes[10], generating and maintaining mechanisms of loss of synchronism in atrial mobility or arrhythmogenic movements[11]. However, although evidence has been found linking fibrosis with atrial fibrillation, the cause-effect relationship between them is not clear yet[12]. Therefore, a more detailed study of these cells of atrial fibrosis and their effect on electrical conduction patterns in atrial tissue is required. Since there is a need to first, establish if there is a relationship between the presence of fibrotic cells (fibrocytes, fibroblasts, myofibroblasts) and the presence of AF and then, measure their effect on the electrical conductivity of cardiac wall cells (cardiomyocytes). In this study, we present some techniques for the co-culture of fibroblasts and cardiomyocytes with different geometric patterns, intending to observe the interaction between these cells and how they influence the electrical activity of the tissue in monolayer arrays.

MATERIALS AND METHODS

Study area: The study was carried out at the Department of Medicine, Systems Biology Research Lab, Bolivarian Pontifical University, Medellin, Colombia from January, 2019 to November, 2020.

Methodology: In the present study, a cell culture protocol was developed using cell patterning techniques[13]. The method consists of creating linear and dot patterns on a wax paper "mask" to establish differentiable regions for a specific cell type (fibroblasts or cardiomyocytes).

Fibroblasts from the NIH-3T3 cell line were used, which corresponds to NIH swiss albino mouse embryonic cells isolated at the Department of Pathology of the New York University School of Medicine in 1962. Due to their adherent morphological characteristics and a duplication time between 20 and 26 hrs, they have become one of the most widely used cell lines in research (e.g., RNA interference, genic expression, gene silencing). The other cell type used in this work was cardiomyocytes of the HL-1 cell line, which correspond to immortalized mouse cardiomyocytes that can continuously divide and spontaneously contract while maintaining a differentiated cardiac phenotype. These HL-1 cells have been used to study normal cardiomyocyte function concerning signalling, electrical, metabolic and transcriptional regulation, as well as to investigate other pathologies such as hypoxia, hyperglycemia, hyperinsulinemia, apoptosis and ischemia.

The HL-1 line was derived from a subcutaneous AT-1 tumour excised from an adult female C57BL/6J mouse. The parental AT-1 line was originally derived from an atrial tumour of a transgenic mouse in which SV40 large T-antigen expression was activated in atrial cardiomyocytes through the Atrial Natriuretic Factor (ANF) promoter.

Cell cultures: fibroblasts NIH-3T3: Cells were cultured under aseptic conditions in T25 flasks containing 5 mL of complete DMEM medium (Lonza) with 10% fetal bovine serum (Gibco) and 500 µL of antibiotic (streptomycin, 1000 µg mL^{-1} and penicillin, 1000 U) (Microgen Ltd.). Cells were maintained in a humidified atmosphere of 95% air with 5% CO_2 at 37°C[14].

Preparation of Parafilm patterns for co-culture of fibroblasts with HL-1 cardiomyocytes: Geometric patterns were designed on paper and then constructed on wax paper (Parafilm® M, P7793, Merck) cut according to the size of the plates used. Then, the pieces of wax paper of approximately 35 mm in diameter (9.6 cm^2 area) with the elaborated patterns were attached to the bottom of plates of 6 wells (Fig. 1).

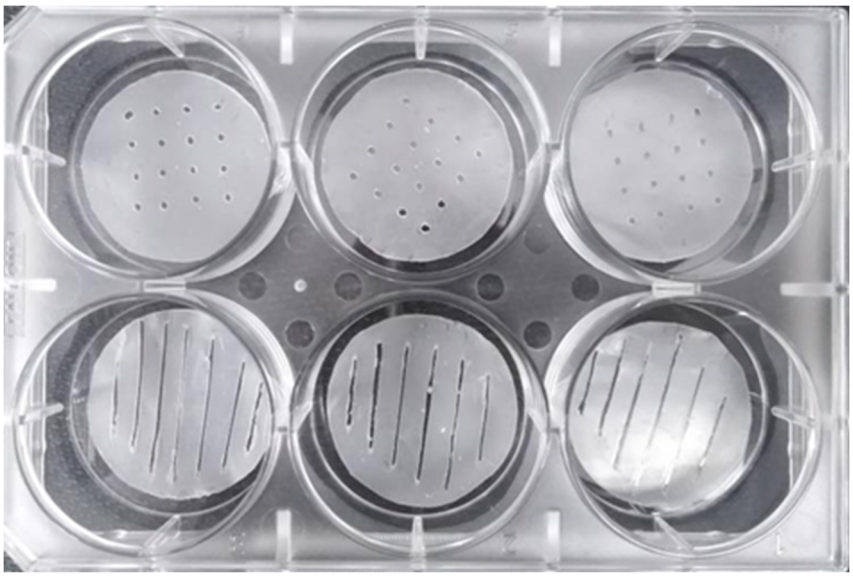

Fig. 1: Dot and line patterns on Parafilm® at the 6-well plates

For dot and linear patterns, a sterile 21G (0.8 mm) hypodermic needle and two sterile scalpel blades were used, respectively. The scalpel blades were coupled so that the space between their edges established the width of the line, achieving an approximate thickness, of 0.8 mm also. Once these patterns were designed, they were sterilized in a Telstar Bio II A laminar flow cabinet (Terrasa, Barcelona and ESP), first by placing them in each well-plate with 5 mL of 70% ethanol for 20 min. After this, the ethanol was removed from the plates and subjected to UV radiation for 15 min. The plates were organized in a way that each had three wells with dot patterns and three wells with linear patterns (Fig. 1) and were stored in sterile conditions with the patterns attached for later use. Sterility tests were carried out for each of these standards.

Fibroblasts proliferation in plates with parafilm patterns: After sterilization of the plates with the patterns, for each well, 50,000 fibroblasts were seeded with 2 mL of Claycomb (Sigma) complete medium, prepared according to the protocol of the supplier of the HL-1 cells. A photographic record was taken 24 hrs later and the patterns were removed with sterile forceps taking care not to affect the lines or dots now occupied by fibroblasts. A new photographic record was taken at 48 hrs to observe proliferation and propagation beyond the patterns (this experiment was done at three independent times and in duplicate).

Fibroblast proliferation in fibronectin/gelatin-treated plates: A fibronectin/gelatin solution (Sigma) was prepared according to the following protocol from the HL-1 cell supplier: 40 mL of ultrapure water and 10 mL of 0.1% gelatin solution (this was previously prepared by dissolving 100 mg of gelatin in 100 mL of ultrapure water with agitation and at 80°C) were added to a 50 mL tube. Then, 500 µL of fibronectin (1 mg mL^{-1}) was added to the solution to obtain a final fibronectin concentration of 5 µg mL^{-1}.

Using this gelatin solution, the plates were treated as follows: In the flow cabinet, the parafilm patterns were momentarily removed from the sterilized plates and 1 mL of the fibronectin/gelatin solution was added to each well, spreading it over the entire bottom of the well with a gentle movement. Then, these covered plates were placed in an incubator at 37°C for 1 hrs. Later, the excess solution was removed with a pipette and the parafilm patterns previously removed and conserved in sterile conditions in the cabinet were put back in. The first experiment was repeated with mixed patterns (dots and linear) but in this case, the fibronectin/gelatin solution was added to each well.

Culture of primary mouse cells: Before the use of HL-1 cells, primary cultures were made from sacrificed animals. Ten BALB/c mice of 3 weeks old were euthanized at different times by cervical dislocation under aseptic conditions (CIB Bioterio cabinet, Medellín). The heart was extracted and the ventricular sections were separated into small pieces to make explants using sterile surgical instruments. These pieces were then seeded in T25 culture flasks with 5 mL of Claycomb complete medium. As a second alternative, mouse or rabbit cardiomyocytes were extracted by enzymatic digestion using the Langendorff retrograde heart perfusion technique, based on the following protocol: a fresh heart (no more than half an hour after extraction from a sacrificed animal) was subjected to retrograde perfusion with Tyrode buffer solution in a progressive manner and with decreasing concentrations of calcium, in the form of $CaCl_2$ (0.5, 0.1 and 0 mM). Then an enzyme solution (proteases and collagenase) was passed through the heart, three times with the same perfusion equipment and the process was repeated in reverse with the Tyrode solution but this time with ascending concentrations of calcium[15]. Finally, the cardiac muscle was placed in a Petri dish with Tyrode solution and with a non-sharp instrument, it was dispersed into pieces so that the cardiomyocytes were released. With this technique, it was possible to observe rabbit cardiomyocytes in the Tyrode buffer medium still with their mechanical activity, but this was only observed for a couple of hours, so the technique was not an option for the isolated cultures or the co-cultures with fibroblast.

HL-1 cell line culture: The HL-1 cell line was kindly donated by the Cell Biology Laboratory of the University of Santander. Briefly, the cells were thawed and then cultured in T25 flasks in Claycomb complete medium and then subcultured in 6-well plates without parafilm patterns, but with prior treatment on their surface with fibronectin/gelatin solution. About 4×104 cells were seeded in each well with 4 mL of Claycomb complete medium and a photographic record was taken at 24 hrs.

Optical mapping and recording of cardiomyocytes: An optical mapping protocol was performed according to Pucihar *et al.*[16] to observe and measure the electrical activity of cardiomyocytes. Before optical mapping, the cells were prepared as follows:

- The culture medium was discarded from the plate
- Each well was two times washed with 3 mL of Tyrode solution
- Four mL of Tyrode solution and then 35 µL of Di-4-Anepps dye (voltage-sensitive marker) were added under dark conditions and the plate was covered with aluminium foil
- The light-protected plate was left in a NuAire incubator (Plymouth, MA, USA) at 37°C for 10 min to allow fixation of the dye on the cardiomyocyte membrane
- Then, under dark conditions, the Tyrode and dye solution was removed and 4 mL of Tyrode solution was added. The plate was covered and taken to optical mapping

For optical mapping, a SciMedia Ltd. electro stimulator (Costa Mesa, CA, USA) was used with electrodes coupled to the plate with the cells, in bipolar (with the two electrodes located at a short distance) and unipolar (one electrode assuming the other at a large distance) conditions. Using a computer, coupled to the electro-stimulator, the electrical pulse was synchronized with the photographic record from the microscope

Fibroblast and HL-1 cardiomyocytes co-cultures: To observe the electrical conduction patterns of both isolated HL-1 cardiomyocytes and co-culture with fibroblasts, optical mapping was performed using Claycomb complete medium (basal medium with 10% FBS+norepinephrine 1%+L-Glutamine 1%+antibiotic 1%).

Briefly:

- Isolated cardiomyocytes mixed with parafilm patterns in cultures plates were treated with fibronectin/gelatin solution and Claycomb complete medium. Then, photographic records were taken at 24 and 48 hrs and optical mapping was performed without the patterns at 48 hrs
- Co-cultures of cardiomyocytes and fibroblast in a 50/50 ratio without parafilm patterns in covered plates were treated with the fibronectin/gelatin solution and Claycomb complete medium. A photographic record was taken and optical mapping was performed at 24 hrs
- Co-cultures of cardiomyocytes and fibroblast with parafilm patterns in covered plates were treated with fibronectin/gelatin solution and Claycomb complete medium. The cells were cultured in different cardiomyocytes/fibroblast ratios: 90/10, 80/20, 70/30, 60/40 and 50/50

RESULTS

Parafilm patterns for co-culture of fibroblasts and HL-1 cardiomyocytes and fibroblasts proliferation in plates with parafilm patterns: Figure 1 shows the photographic record of a 6-well plate disposed to the experiment with both linear and dots parafilm mixed patterns. The proliferation of the fibroblasts in the plates with parafilm patterns without and with fibronectin and gelatin is shown in Fig. 2a-c.

Cardiomyocyte proliferation in fibronectin/gelatin-treated plates: In the primary mouse heart cultures, it was observed that after several days cells were expanding around the heart pieces (Fig. 3a), but most of them were fibroblasts and some few cardiomyocytes. Cardiomyocytes do not survive long and the medium is quickly invaded by fibroblasts. Because there was not only a single cell type, it was sought to separate them without success even with flow cytometry because the differentiation was not very noticeable in size and complexity and no and no fluorescent membrane markers were available for this study.

The HL-1 cells were sub-cultured with Claycomb complete medium and then seeded in fibronectin/gelatin-treated plates. Figure 3b shows a microscopic view of the HL-1 cardiomyocytes growing in a plate with a dot parafilm pattern. Linear and dots parafilm patterns were placed in other wells of the plate and 40.000 HL-1 cardiomyocytes were seeded. After 48 hrs, the cultures were observed under the microscope and it could be noticed that most of the HL-1 cells grew on the surface of the plate and a few on the parafilm surface without fibronectin/gelatin.

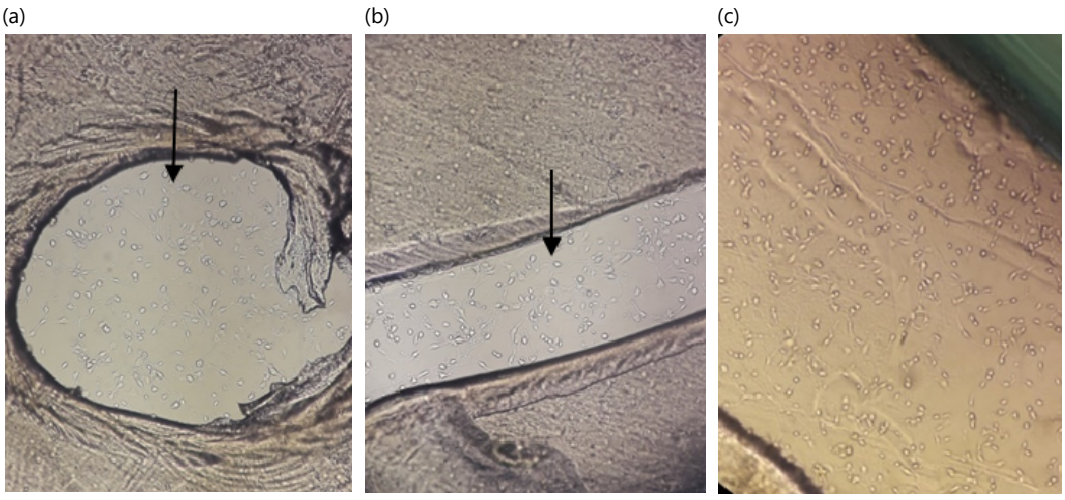

Fig. 2(a-c): Fibroblast proliferation in plates with parafilm patterns
Fibroblast proliferation without fibronectin and gelatin (a,b) and fibroblast proliferation in fibronectin/gelatin-treated plates with parafilm patterns (c). The black arrow shows the growth of fibroblasts within the patterns

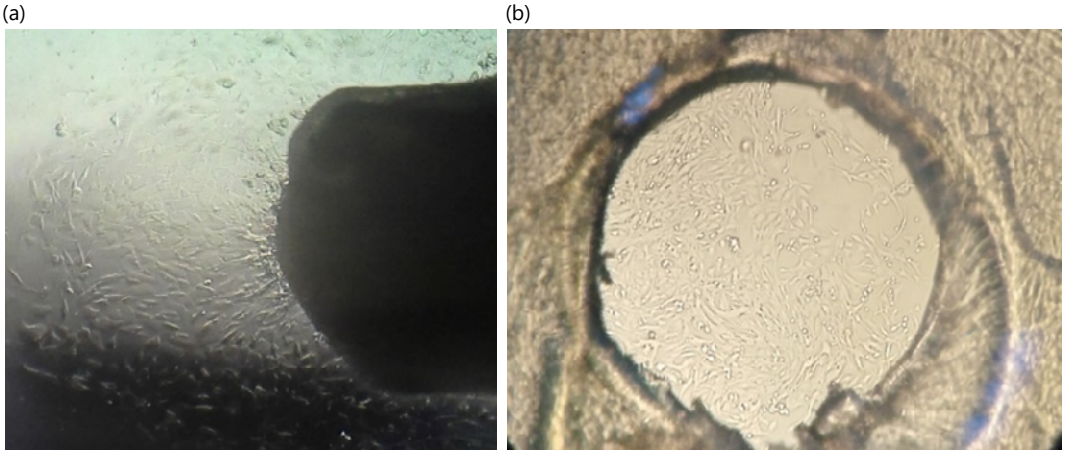

Fig. 3(a-b): Microscopic view of primary cultures of mouse ventricular cells (a) and HL-1 cardiomyocytes in culture medium and with parafilm dot pattern (b)

Cardiomyocytes extracted by enzymatic digestion: Using enzymatic digestion, the mechanical activity of rabbit cardiomyocytes could be observed for approximately 20 min. However, neither their electrical activity nor their interaction with other cells could be measured due to the low confluence (<25%) and the short half-life time, which was insufficient to set up the mapping equipment.

DISCUSSION

Tissue functionality is directly dependent on the interactions that occur between the cells that make up the tissues and their extracellular matrix, which guarantees the normal development and functioning of the tissue. Some disorders in the development of such interaction processes are associated among other things with the appearance of fibrosis[17], which in turn appears to be related to the development of atrial fibrillation. Due to this, being able to establish *in vitro* models that can simulate pathological patterns of cardiac structure is of utmost importance to understand the different aspects responsible for the appearance of such disorders in the tissue[18,19].

In this study, rat fibroblasts of the NIH-3T3 line were cultured in plates with a complete culture medium, parafilm patterns or fibronectin/gelatin solution added. These cells had a good response in terms of growth and proliferation both in the medium and in plates with the protein (fibronectin/gelatin solution), reaching confluences close to 100%, as expected as reported by Franco-Barraza *et al*.[20].

In primary cultures, there was a remarkable proliferation of mouse ventricular cells but it cannot be distinguished between fibroblast and cardiomyocytes, so the experiment by this route was not suitable for the study. Nevertheless, although it was possible to observe the HL-1 cells in subcultures, it was difficult to measure its mechanical activity by optical mapping because toxicity was evidenced with the different Di-4-Anepps dye concentrations used. This photodynamic damage effect caused by this dye on isolated cardiomyocytes has been reported previously[21,22], so, it is necessary to test other methods for optical mapping. Additionally, to observe this activity, cells are required to be previously stimulated with norepinephrine and in a high confluence, because the proximity between cells allows the action potential propagation and thus observe an electrical conduction pattern.

Although cells resulting from the enzymatic digestion according to the most used protocols[23-25] have activity, which characterizes them as cardiomyocytes, their short half-life does not allow realized subcultures to obtain them separately and in a controlled manner for co-culture with fibroblasts.

CONCLUSION

It is concluded that cultures of heart fibroblasts can be grown in DMEM and Claycomb medium and do not require fibronectin protein to grow up, whereas cardiomyocyte-like cells require specific Claycomb medium and norepinephrine to maintain their electrical activity and required to be cultured in a flask previously treated with a fibronectin/gelatin solution. A confluence close to 100% has to be guaranteed to observe any effect on the conduction of the action potential.

SIGNIFICANCE STATEMENT

Atrial fibrillation (AF) is a cardiac arrhythmia, which consists of the presence of chaotic and unsynchronized contractions due to the presence of arrhythmogenic fibrotic tissue. Due to the high public health impact of atrial fibrillation, studies like this one, with a comparison of the conduction patterns obtained in co-cultures of fibroblasts and cardiomyocytes as observed in *in silico* models is required. Through this integration of cardiac tissue experiments with simulation techniques, the formation of interdisciplinary research in the field of cardiovascular medicine can be achieved, with the application of more specific and effective therapies in heart disease, such as the specific ablation of fibrotic cells and the prevention or reversal of fibrosis.

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