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Isolation of Adult Rat Cardiomyocytes Using Recombinant Collagenases

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Direct isolation of primary cells from tissues and organs allows for the maintenance of important cell characteristics and properties for *in vitro* studies and a plethora of biomedical applications. Dissociation of cells from the organ of interest is possible due to the enzymatic activity of collagenases. The choice and the dose of these enzymes is the critical step to obtain the maximum number of cells with intact structure and function. In this contest, Abiel collagenases class I (Col G) and class II (Col H) were synthesised using recombinant DNA technologies and their ability to degrade collagen in cell isolation from different tissues was tested. Examples of cells isolated with these enzymes include Langerhans islets, hepatocytes, chondrocytes, osteoblasts, fibroblasts, and stem and retinal cells that be used for *in vitro* studies. In this contest, primary cardiomyocytes represent a perfect murine model to investigate numerous heart diseases. Herein, a protocol of cardiomyocyte extraction from rat heart, using a combination of Abiel collagenases supplemented with thermolysin protease, is proposed. The structure and the viability of isolated cells were tested over time by optical and fluorescence microscopy and viability assays. Further cellular structure characterisation was performed by western blot analysis, using specific cardiomyocytes markers. Isolation of viable primary heart cells with unaltered properties and functionality can potentially provide *in vitro* models to study heart function, arrhythmias, long Q-T syndrome and cardiotoxicity.

1. Introduction

The use of primary cells directly extracted from organs and tissues is becoming of great interest in the study of human and animal biology. These cells can be used as a more representative model of the original tissue components regarding their functionality respect to continuous cell lines. This has found a plethora of applications in tissue engineering as well as in the study of specific cellular diseases (Rosenthal and Brown, 2007). Collagen is one of the main components of extracellular matrix (ECM) and resists the action of most part of proteases, with the exception of a limited number of collagenolytic proteases. Selecting and optimising the concentration of the appropriate collagenases is of great importance to degrade the ECM releasing the cells from their original tissue and obtaining a good yield of isolated functional cells. Commercial collagenases are produced in large amount by the gram-positive bacterium *Clostridium hystoliticum*. It is pathogenic in many species, including guinea pigs, mice, rabbits, and humans; therefore, in many cases, the direct collagenases production is considered to be not well appropriate. Therefore, new pharmaceutical standards require the use of a safer host like *E. coli*. In this contest, Abiel collagenases class I (Col G) and class II (Col H) were synthesised in fermentation using recombinant DNA technologies in *E.coli* vector and then purified by chromatography (Volpe et *al.*, 2016)
These enzymes were successfully used in the extraction of different types of cells including Langerhans islets (Salamone et *al.*, 2010), hepatocytes, chondrocytes, osteoblasts, fibroblasts, stem and retinal cells (Salamone et *al.*, 2014). Therefore, isolated cells are suitable for experiments aimed at visualising cellular components and functions or to study specific localisation of intracellular molecules.

In this regard, cellular studies of primary cardiomyocytes can widen the scope of cardiac physiology and cardiac disease. Isolated cardiomyocytes are commonly used in the study of intracellular Ca^{2+} homeostasis, contraction of myofilaments and all the calcium-dependant cellular mechanisms associated and protein biochemistry. Furthermore, they can easily be infected or transfected for gene transfer studies (Louch et *al.* 2011). Some applications of isolated cardiomyocytes from transgenic mouse and rat models include the study of cardiac alterations due to protein malfunction of the contractile apparatus within single cells (Sussman et *al.*, 1998) and viability and analysis of cell behaviour in disease models like ischemia or hypertrophy (Heinzel et *al.*, 2005; Li et *al.*, 2005). Therefore, the combination of the information provided by *in vitro* models and *in vivo* experiments in animal models or intact tissue can provide an improved examination of cardiac function in a hierarchical manner.

The method used for cardiomyocyte isolation is thus of critical importance to obtain a good yield of extracted cells preserving their *in vivo* integrity and function. Herein, an isolation protocol of adult rat cardiomyocytes using Abiel recombinant collagenases is proposed. This protocol consists of two different digestion steps aimed to increase the culture purity and the possibility to obtain single cells. Characterisation was carried out in the presence of cardiac specific markers Troponin I and alpha-actinin.

2. Method

2.1 Isolation and culture of rat cardiomyocytes

Abiel collagenases class I (Col G) and class II (Col H) were synthesised by recombinant DNA technologies in *E.coli* vector as descripted in Volpe et *al.*, 2016.

Cardiomyocytes were isolated from female Winstar rats with a weight varying between 150-200 g using a protocol of two-step collagenase perfusion. Once the animals were anesthetised, heart was extracted during the surgery. A cannula was placed in the aorta cavity and connected to a perfusion system linked to a peristaltic pump. After an initial wash of the heart cavities with perfusion buffer (NaCl 120 mM, KCl 5.4 mM, Na2HPO4*7H2O 0.33 mM, MgSO4*7H2O 0.5 mM, BDM 10 mM, HEPES 25 mM, Glucose 22 mM, insulin 0.05 mU/l, 0.4 mM EGTA), a second perfusion was performed for 25 minutes in recirculating mode using a digestive buffer (Collagenases G 1.5 U/ml; Collagenase H 7.5 U/ml (Abiel s.r.l.); Thermolysin 10 µg/ml). Once the first digestion was completed, the heart was transferred to a sterile petri dish and a second digestion step was carried out. Fresh digestion buffer was added and the heart was quickly triturated with fine tweezers and forceps. This second digestion was performed at 37°C in an incubator with 5% CO_2 for 15 minutes in order to facilitate the collagenase action. The incubation was finished by adding stop buffer containing foetal bovine serum (FBS) (Sigma) and the sample was filtered using a 230 µm mesh. Following this, cardiomyocytes were purified by gravity in a falcon tube for 15 minutes and washed with Minimum Essential Medium Eagle (MEM media). After purification, cells were counted in a hemocytometer, seeded in laminin coated culture dishes and placed in an incubator with 5% CO_2 at 37°C.

2.2 Study of cardiomyocytes morphology

Morphology of extracted cardiomyocytes was analysed by optical and fluorescence microscopy on the following days after isolation. To perform fluorescence microscopy, cells were seeded into laminin-coated 12-well plates containing sterile coverslips and grown in complete medium. Cells were fixed with 3.7 wt % formaldehyde for 10 minutes and permeabilised using Triton X-100 at concentration of 0.01% in phosphate buffer saline (PBS) for 5 minutes. After three washes with PBS, nuclei were labelled with DAPI (1: 10000) for 15 minutes at 37°C. Plates were incubated at 4°C overnight with anti alpha-actinin antibody produced in mouse (1:1000 in PBS) and anti-troponin antibody produced in rabbit (1:600 in PBS). Secondary antibodies were then incubated for 2 hours at 37°C: anti-mouse FITC-conjugated (1:1000 in PBS) and anti-rabbit TRIC-conjugated (1:500 in PBS). Fluorescence signals were detected using a fluorescence microscope (Leica). Untreated cells were used to calibrate the background fluorescence.

2.3 Viability assay

Cardiomyocytes were seeded in laminin-coated 96-well plates and kept at 37°C in 5% CO_2 after isolation. Viability assay was conducted using Cell Counting Kit-8 (CCK-8) (Sigma Aldrich): the water-soluble tetrazolium salt (WST-8) was added at a 1:10 dilution in complete medium on each sample. Subsequently, cells were incubated at 37°C for 2h to allow for mitochondrial dehydrogenases-induced reduction of WST-8 into a soluble formazan dye by the metabolically active cells. Absorbance was measured at λ=450 nm by a microplate reader DU-730 Life Science spectrophotometer (Beckman Coulter). Each experiment was done in triplicate.

2.4 Extraction of Soluble Proteins and Western Blot Analysis

Cardiomyocytes were seeded on six-well plates and kept at 37°C in a humidified atmosphere of 5% CO_2. 1 and 3 days after isolation, cells were washed with PBS without Ca^{2+} and Mg^{2+}, detached by Trypsin-EDTA 1X in PBS (EuroClone) and centrifuged at 1000 rpm for 5 minutes. Pellets were resuspended in an appropriate volume (depending of the pellet size) of RIPA Buffer 1X (50mM Tris-HCl pH 7.5; 0.5% sodium deoxycholate; 150 mM NaCl; 1 % Triton X-100). Subsequently, samples were incubated in ice for 15 minutes whilst subjected to micro vortex mixer treatment. The extract was centrifuged (10,000 rpm for 20 minutes) at 4°C to remove insolubilised proteins and cellular debris. The amount of proteins extracted was calculated using a Bradford assay (Bradford Reagent, Sigma).
10 µg of proteins were mixed with sample buffer 1X (62.5 mM Tris-HCl pH 6.8; 2.5 % SDS; 0.002 % Bromophenol Blue; 0.7135 M (5%) β-mercaptoethanol; 10 % glycerol) and incubated for 5 minutes at 100°C. The proteins were resolved using 7.5 % sodium dodecyl sulfate -polyacrylamide gel electrophoresis (SDS-PAGE) and transferred from the gel to a nitrocellulose membrane (Hybond, Amersham) through electroblotting at 100 V and 300 mA for 90 minutes at low temperature, in transfer buffer (20% methanol; 10% Running buffer 1X: Tris base 3 g/L-Glycine 14.4 g/L). Non-specific binding was blocked by placing the membrane in a solution of non-fat dry milk (3%) overnight a 4°C. Troponin I antibody produced in rabbit (1:1000, Thermo Fisher Scientific) was incubated over night at 4°C and, after thorough washing with TBS-T solution (0.6% Trizma base; 0.87% NaCl; 0.05% Tween 20), the peroxidase-linked anti-rabbit secondary antibody (0.04 µg/mL, Sigma Aldrich) was incubated for 2 h at room temperature. After multiple TBS-T washes, Toponin I protein was detected using “SuperSignal West Femto Maximum Sensitivity Substrate” (Thermo Scientific) and chemiluminescence signal was revealed using ChemiDoc XRS (Biorad, Hercules, CA, USA).

3. Results

3.1 Isolation of cardiomyocytes from an adult rat heart

Isolation of primary cells is a process that requires careful optimisation, particularly the nature and the amount of digestive enzymes such as collagenase. Lower doses can reduce the number of extracted cells, whilst higher concentrations can compromise the cell structure/function. Consequently, recombinant collagenases G and H in association to proteases (pronase or thermolysin) were used at different concentrations (Col G: 2-1 U/ml, Col H: 10-5 U/ml, 200 µg/ml pronase or 0.22-10 µg/ml- thermolysin) and with different incubation times, aiming at obtaining the maximum number of functional cells. The best extraction was obtained with 1.5 U/ml of Col G, 7.5 U/ml of Col H and 10 µg/ml of thermolysin. Digestion of ECM was carried out in two steps. The first step was performed in a circulating system to partially degrade collagen and its mechanical support function. The second step was performed after a mechanical trituration of the heart, incubating the small fragments at 37°C in a digestion solution. This allowed the enzymes to isolate single cells from the tissue mass. With this protocol, we obtained approximately 3 million cells from an adult rat heart, subjected to individual variability. Extracted cells presented good morphology, as shown in Figure 1. Typical rod-shaped morphology with sharp distinct edges was evident under bright-field illumination. It was also possible to identify the characteristic calcium-dependent contraction of actin-myosin complexes (data not shown).

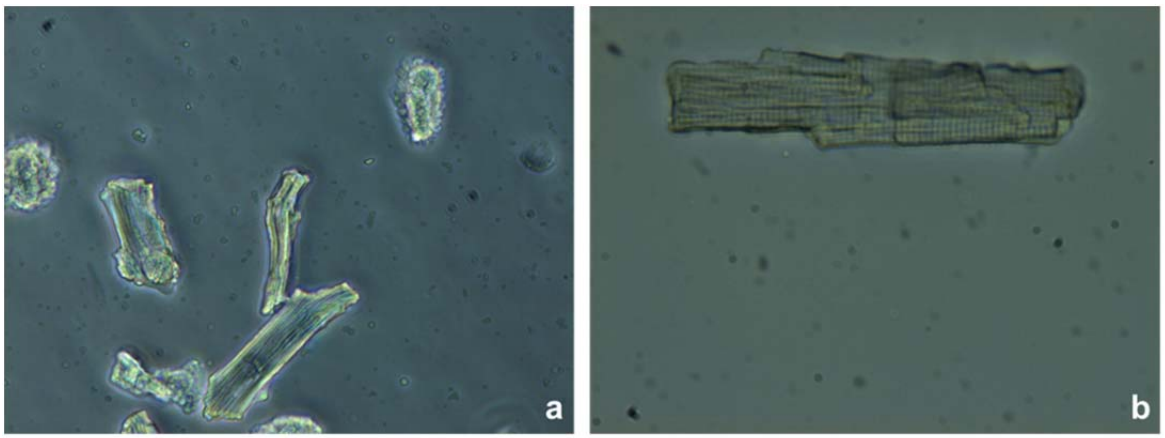

Figure 1. Cardiomyocytes isolated from an adult rat heart. Optical microscopy. Magnificence: 20X (a) and 40X(b).

3.2 Morphological cardiomyocytes characterization

One of the predominant characteristics of cardiac cells is their capability to contract myofilaments in a calcium-dependent manner. Following an electrical stimulus, calcium can enter cells through voltage gated channels of the plasma membrane and causes calcium release from the sarcoplasmic reticulum (SR), increasing its cytoplasmic concentration. Therefore, it binds to troponin C, with the subsequent enhancement of the interaction between myofilaments proteins actin and myosin. Alpha-actinin plays an import role in this machinery as it bonds to the actin filament, stabilizing the muscle contractile apparatus (Woodcock and Matkovich,2005).
Consequently, morphology of extracted cardiomyocytes was analysed by fluorescence microscopy, using specific antibodies against troponin I and alpha-actinin. The specific complex antibody-protein was then detected using the secondary antibodies anti-mouse FITC –conjugated for alpha-actinin antibody and anti-rabbit TRIC-conjugated to troponin. As shown in figure 2, cells presented the typical distinct sarcomeric banding pattern. In conjunction with morphological analysis and nuclear staining with DAPI, immunostaining can be used to identify the purity of the isolated cardiomyocytes. 1 day after seeding (Figure 2a), cells were attached and maintained a predominantly rod-shaped appearance with intact sarcomeres. Higher numbers of rod-shaped cardiomyocytes are a good indication of successful perfusion and digestion processes. Generally, viable adult cardiomyocytes are 100-200 µm in length. A lower percentage of rod-shaped cardiomyocytes suggests an unproductive isolation, probably due to incorrect cannulation of aorta or an inefficient perfusion due to the presence of air bubbles. After 3 days (Figure 2b), some cells become rounded while others still maintain the rod-shape. A small number of cells were not initially attached to the laminin substrate. In subsequent days, attachment improved and cell migration started to occur (data not shown).

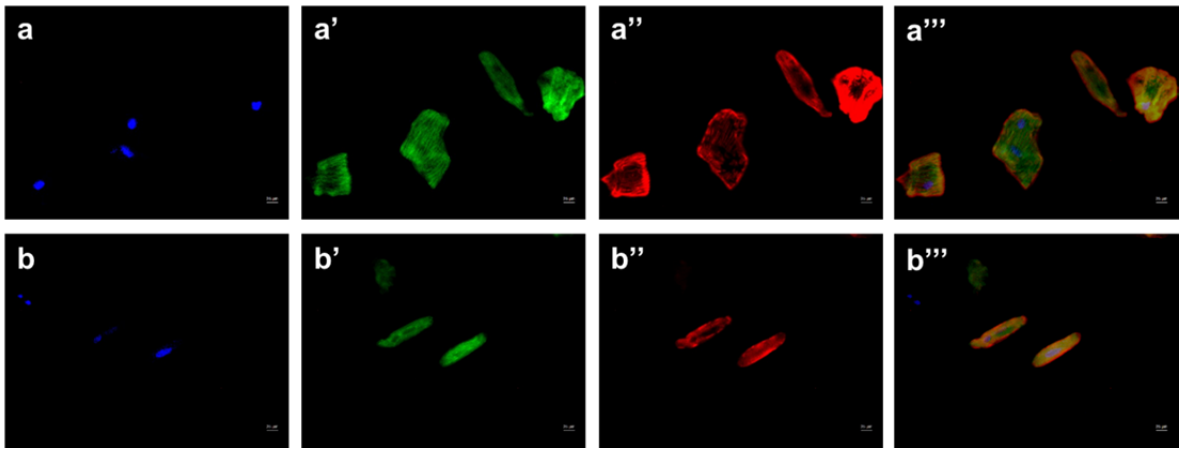

Figure 2. Fluorescence microscopy of cariomyocytes seeded after 1 day (a) or 3 days (b) from the isolation. Blue: nuclei; Green: alpha-actinin; Red: Troponin; merge: overlay of three fluorescence. Magnificence 40X.

Further characterisation was performed by measuring troponin I expression through western blot analysis of total protein extracted on day 1 after cell isolation (Figure 3). Band corresponding to Troponin I protein was evident, suggesting that isolated cells express high level of this protein, as it is typical of cardiac cells.

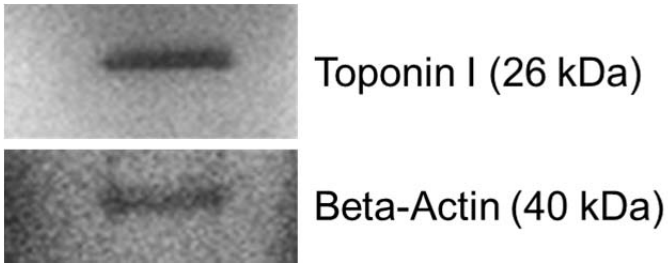

Figure 3. Western Blot analysis of troponin I expressed in cardiomyocytes cells after 1 day from the isolation. Beta-actin was used as control.

3.3 Viability of extracted cardiomyocytes

A good isolation protocol requires utmost care during cannulation of the aorta and digestion phases. These processes can heavily influence the viability of the cells during the following days after extraction. Generally, a successful isolation and culture harvest approximately 50% of fully-functional cardiomyocytes after few days (Judd et *al.*, 2016).
Viability assay was performed on cardiomyocytes after day 1 and day 3 from the extraction (Figure 4). Number of cells decreased ca. 40% on day 3 as expected, suggesting a good extraction.

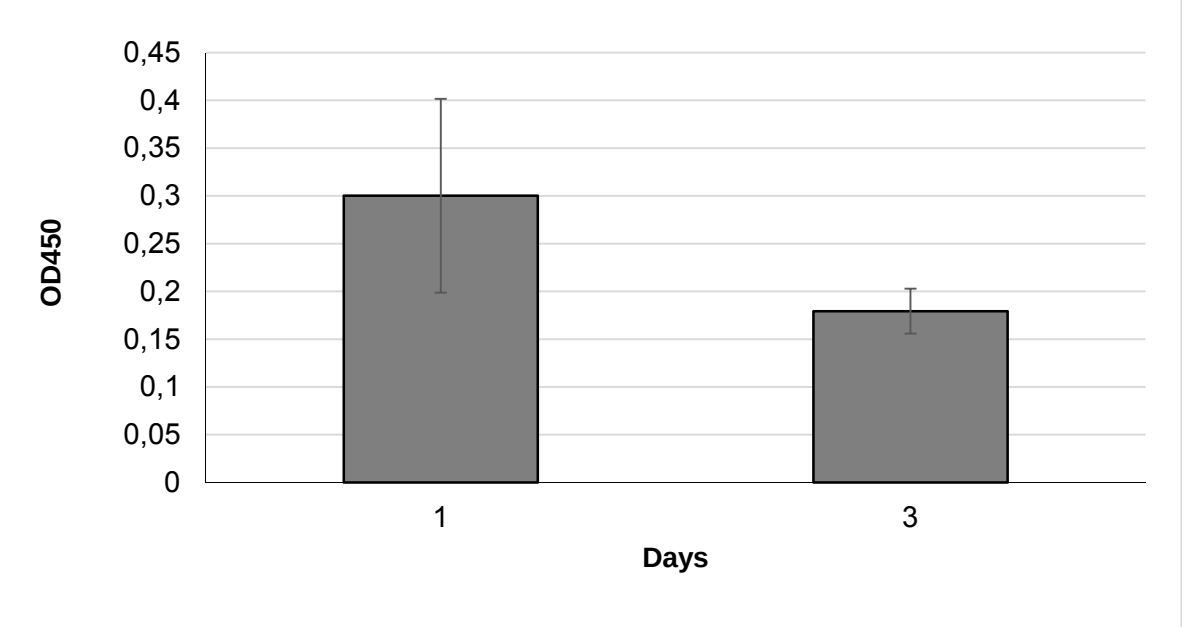

Figure 4. Cell viability assay on cardiomyocytes after 1 and 3 days from extraction. Each experiment was done in triplicate.

4. Conclusion

Culturing primary cardiomyocytes directly extracted from rats or mice, allows for advantages in the study of diseases produced by alterations in protein expression, for in-depth analysis of electrophysiology, intracellular calcium fluxes and contractile mechanics. Therefore, the extraction protocol requires careful execution in order to obtain the maximum number of functional cells. Selection and concentration of enzymes with collagenolytic activity and the digestion time play a fundamental role in obtaining single cells that maintain their properties for a suitable period after extraction. Respect to the commercial collagenases derived from the pathogenic bacterium *Clostridium hystoliticum*, recombinant Abiel collagenases are produced in safer bacterium *E.coli* and are purified by chromatography. This process permits to obtain enzymes with a high purity grade, without any toxic contaminants and more stable respect to the commercial ones. In particular, simultaneous action of class I and class II Abiel collagenases, in association with thermolysin, permits to isolate cells from different tissues with the unaltered morphologic and physiologic properties (Salamone et *al.*, 2010). The use of these recombinant collagenases had permitted us to extract cardiomyocytes with the characteristic roughly rod-shaped morphology with rectangular ends and clear cross-striations. Cells also showed the presence of specific markers like troponin and alpha-actinin, as demonstrated by fluorescence microscopy and western blot analysis. The protocol herein proposed allows for the isolation and culture of viable and functional cardiomyocytes, suitable for a number of purposes such as therapeutics for human disease.

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