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Development of a Method for Isolation of Mature Cardiomyocytes from Human Heart Biopsy Specimens

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To increase the yield of living cells and their survival, studies were carried out to optimize the method for isolating cardiomyocytes from biopsy specimens excised from the right atrial appendages. It was found that creatine, blebbistatin, and taurine are necessary components of the buffer solution during cardiomyocyte isolation, and that composition of the solutions is a more important factor than their oxygenation.

Key Words: *isolated human cardiomyocytes; method of isolation; electrophysiology; immunocytochemistry on a confocal scanning microscope*

Before discovery of induced pluripotent human stem cells (iPSC), animal cardiomyocytes were used as experimental cardiomyocyte culture model; however, this model cannot adequately reproduce the function of human heart cells, and consequently, human heart tissue due to differences in the composition of ion channels in human and animal cardiomyocytes [1-3]. At present, cardiomyocytes differentiated from iPSC actively replace animal cell model [4-6]. The culture of cardiomyocyte cells obtained from iPSC can form a monolayer that conducts electrical excitation and can be used to study the causes of arrhythmias and antiarrhythmic means. Moreover, the monolayers and isolated cardiomyocytes differentiated from iPSC can be used for testing drug cardiotoxicity. However, the question on the degree of maturity of these cells remains open [7,8]. The results obtained at the Research Laboratory of Molecular and Cellular Diagnostics of the M. F. Vladimirsky Moscow Region Research Clinical Institute suggest that electrophysiologically cardiomyocytes from iPSC reach maturity by day 30 from the beginning of differentiation [9].

To study the factors of cardiomyocyte development, it seems useful to compare functional properties of cells obtained by differentiation from iPSC and mature human heart cardiomyocytes from myocardial specimens obtained during surgery (biopsy) [10-12]. Isolation of cardiomyocytes from a biopsy the human heart is a difficult task. In international practice, various protocols for isolation of mature cardiomyocytes have been developed [13-16]. The general principle is enzymatic digestion of the cardiac tissue to obtain single cardiomyocytes that are studied only on the day of isolation. At the same time, the yield of cardiomyocytes and their survival greatly vary [17]. Based on the experience of foreign colleagues, we have developed and patented a method for isolating cardiomyocytes from human heart biopsy specimens [18].

Here we present an optimized protocol for isolation of mature human cardiomyocytes from biopsy specimens, possible variations of this protocol, and their effects on the yield of living cells.

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MATERIALS AND METHODS

The study was approved by the Ethics Committee of the M. F. Vladimirsky Moscow Region Research Clinical Institute (Protocol No. 13479/2019, February 19, 2019) and carried out in accordance with the WMA Declaration of Helsinki (2004). All patients (mean age 60 years) gave written informed consent for material sampling.

Cardiomyocytes were isolated from the biopsy specimens as described elsewhere [18]. The biopsy material was minced to 1-2 mm fragments in the cold, washed from the cardioplegic solution with a calcium-free buffer for 9-10 min, then incubated in two portions of a solution with an enzyme mixture 37°C; the supernatant was discarded each time. Then, a calcium-free solution with collagenase IV was added to the fragments and incubated for 7-15 min at 37°C and constant stirring. The fluid was carefully collected without agitation. This stage was repeated 3-5 times. After each cycle, the collected suspensions were filtered through nylon mesh with a pore size of 100 μm and then centrifuged at 800 rpm for 1-3 min. After centrifugation, the supernatant was removed, the pellet was resuspended in fresh calcium-free buffer with 5% fetal bovine serum. Then, calcium reintroduction was carried out: calcium ions (1 ml of an aqueous solution of $CaCl_2$ with a concentration 30 mM) were gradually added to the cardiomyocyte suspension (portions of 0.5, 1, 2, and 4 μl every 5 min). After 30 min isolated cardiomyocytes were ready for experiments.

Isolated living cells were counted in a Goryaev's chamber under a microscope. Biopsy sampling, transportation, and electrophysiology are described in detail previously [19,20].

Solutions used. Calcium-free buffer: 126 mM NaCl, 4.4 mM KCl, 5 mM $MgCl_2$, 5 mM Na_2HPO_4, 5 mM HEPES, 22 mM glucose, 20 mM taurine, 5 mM creatine, 5 mM Na-pyruvate, and 10 μM blebbistatin.

Extracellular solution for $I_{Ca,L}$ recording: 160 mM TEA-Cl, 5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, 2 mM 4-AP (pH 7.4 CsOH).

Intracellular solution for $I_{Ca,L}$ recording: 145 mM CsCl, 5 mM NaCl, 2 mM $CaCl_2$, 5 mM EGTA, 10 mM HEPES, 5 mM Mg-ATP (pH 7.2 CsOH).

Extracellular solution for I_{Ks} recording: 150 mM NaCl, 5.4 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 15 mM glucose, 1 mM Na-pyruvate, 0.001 mM nisoldipine, 0.001 mM E-4031, 15 mM HEPES (pH 7.4 NaOH).

Intracellular solution for I_{Ks} recording: 20 mM KCl, 5 mM Mg-ATP, 10 mM EGTA, 125 mM K-aspartate, 1 mM $MgCl_2$, 2 mM Na_2-phosphocretatin, 2 mM Na_2-GTP, 5 mM HEPES (pH 7.2 KOH).

Cardiomyocyte seeding on slides. Coverslips (12 mm) were prefired with 90% ethanol, placed in 24-well plates, and subjected to UV irradiation in a laminar. The slides were then coated with laminin (rhLaminin-521; Gibco) in a concentration of 10 μg/ml, diluted with PBS, and placed in an incubator for 30 min. After that, the suspension was added so that it completely covered the coverslips with laminin.

Immunocytochemistry. Detailed staining protocol is presented elsewhere [21]. Cell morphology was studied by staining for F-actin (Alexa Fluor 488 phalloidin) that is present in both cardiomyocytes and fibroblasts. For cell identification, the cells were stained for α-actinin, a protein of cardiomyocyte contractile apparatus (mouse monoclonal antibody to α-actinin (sarcomeric), 1:1000; Alexa Fluor 594 (ab')2 goat anti-mouse IgG fragment (H+L), 1:1000; Sigma). The cell nuclei were stained with DAPI (Invitrogen).

Stained atrial cardiomyocytes from the biopsy specimens were examined under a confocal LSM 710 microscope (Carl Zeiss) using a Zen 3.0 black software (Carl Zeiss).

Statistical analysis. The data were processed using Clampfit 10.2 (Molecular Devices) and OriginPro 8.1 software (Originlab Corporation). The data of patch-clamp experiments (current-voltage characteristics normalized to cell capacitance) are presented as *M*±*SD* (for at least three cells from the specimens of different patients).

RESULTS

Isolation protocol variations. To refine the protocol and variants of some its stages, we used the cells isolated from 103 biopsy specimen. The cell yield was considered perfect and sufficient if the number of rod-shaped atrial cardiomyocytes (Fig. 1) after isolation was at least 1.5×10^6/g biopsy material, which was the average level for isolation in laboratory settings according to the described protocol.

The optimal size of the excised biopsy specimen is 1×1×0.5 cm. Too large specimens are often ischemic, while the cardiomyocyte yield from small specimens is low.

The general scheme of isolation steps and some variations tested in the Research Laboratory of Molecular and Cellular Diagnostics, M. F. Vladimirsky Moscow Region Research Clinical Institute are shown in Figure 2. During surgeries with Normacor cardioplegic solution, it is often mixed with blood, so the stage of biopsy transportation was carried out at different blood–solution proportions (Table 1). Cardioplegic solution Custodiol during perfusion is not mixed with the blood, so the mixtures with the blood was not used. After transportation of the biopsy in Normacor–blood (1:2) mixture followed by cardiomyocyte isolation according to the optimized protocol, cardiomyocyte

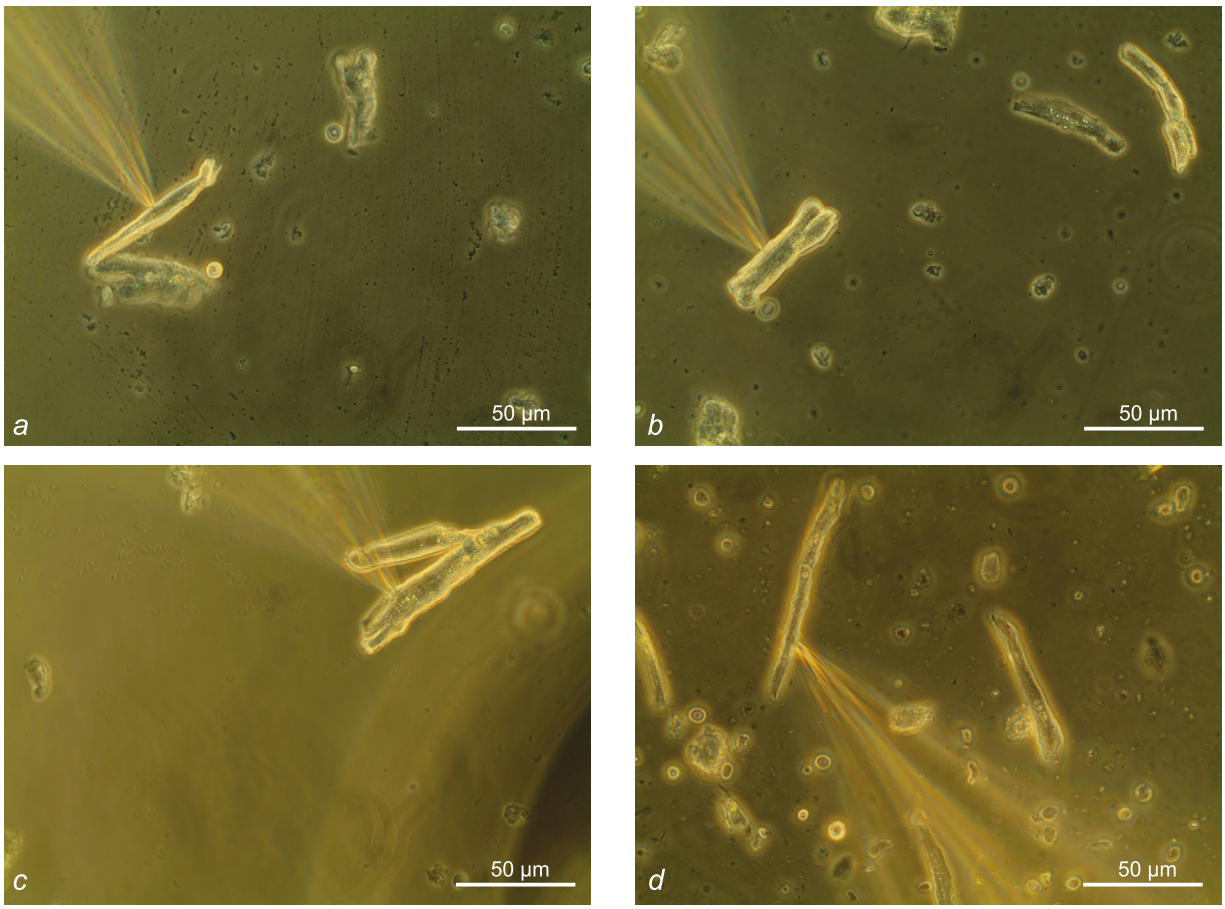

Fig. 1. Atrial cardiomyocytes isolated from different biopsy specimens.

isolation failed in all cases (0 out of 4 isolations). At the Normacor–blood 1:1 ratio, 1 out of 5 procedures was successful. At the Normacor–blood 2:1 ratio, successful isolation of cells was achieved in 40% cases. The highest percentage of successful isolations (80%) was achieved after transportation in pure Normacor. Transportation was carried out at 4°C to slow down the metabolic processes.

Washing the biopsy fractions 3 times for 5 min in oxygenated calcium-free buffer increased the proportion of successful cardiomyocyte isolations compared to 3 times washing for 3 min [18].

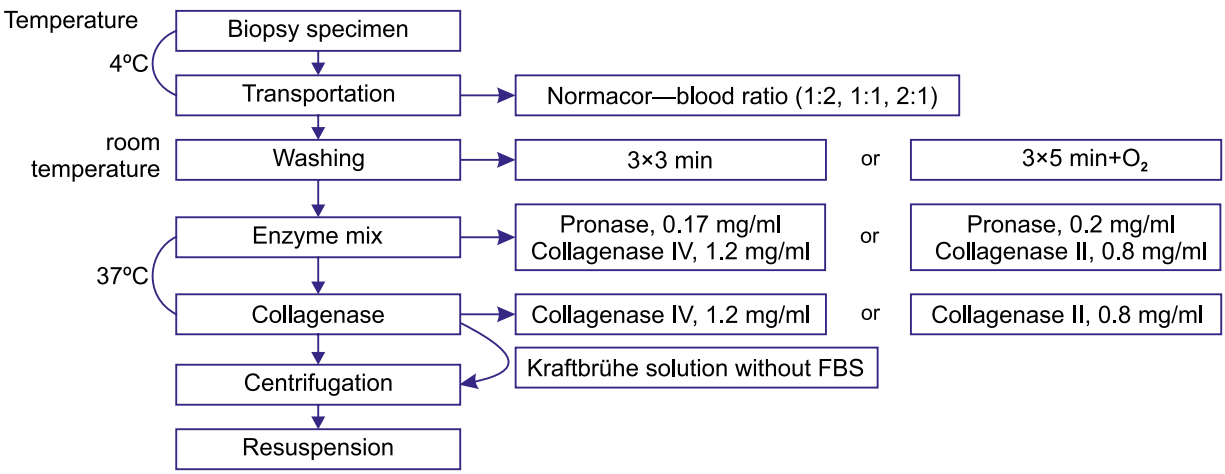

Fig. 2. General scheme of cardiomyocyte isolation from biopsy specimen of the right atrial appendage with variations.

TABLE 1. Transportation in a Solution of Normacor with Blood

Normacor:blood ratio	Number of isolations	Number of successful isolations	% of successful isolations
1:2	4	0	0
1:1	5	1	20
2:1	5	2	40
Without blood	10	8	80

During cardiomyocyte isolation from the biopsy sample, type II collagenase (Gibco) was tried as an alternative to type IV collagenase used in the patented method [18]. The mean concentration of collagenase IV was 1.2 mg/ml; as activity of collagenase II is higher, its concentration was 0.8 mg/ml (in this case, the optimal concentration of pronase when using collagenase II was 0.2 mg/ml).

In patients under 50 years, the heart tissue was more elastic and rigid, therefore, for successful isolation of cardiomyocytes, the time of enzyme exposure was increased to 7 min. In patients above 50 years, the biopsy was less elastic, so fermentation of fragments took only 2-3 min per cycle.

To determine the optimal composition of the solution, a series of isolations of cardiomyocytes from a biopsy sample was performed in different variants of calcium-free solution and without some components (Table 2). Of 14 isolations without creatine, 9 (64%) were successful. The absence of taurine considerably impaired the efficiency of isolations: 43% of successful isolations. In the absence of blebbistatin, the percentage of successful isolations also decreased to 50%. Isolation procedures in the calcium-free solution without two reagents were also less effective: 40% live cells in the absence of creatine and taurine and 50% without creatine and blebbistatin.

Oxygenation of a calcium-free solution allowed increasing the number of successful cardiomyocyte isolations (to 80%) despite the absence of two reagents, taurine and blebbistatin.

In a series without blebbistatin addition to the calcium-free buffer, but with its oxygenation, 10 of 11 isolations were successful. In the next series, the absence of bovine serum (FBS) in the calcium-free solution reduced the success rate to 88% (Table 2).

Even short-term absence of creatine, blebbistatin, or taurine was critical for cardiomyocyte survival. This is due to the fact that these components are responsible for energy metabolism in cells and suppression of cardiomyocyte contractility to reduce energy expenditure. For instance, creatine plays a key role in heart contraction and energy metabolism [22]. In heart failure, the levels of creatine and phosphocreatine decrease due to reduced expression of the creatine transporter, as well as degradation of phosphocreatine to prevent ATP depletion [23]. Blebbistatin is an inhibitor of actin–myosin interaction in the contractile system of the heart muscle [24]. It is more often used than other uncoupling agents because it

TABLE 2. Variations of Reagents and Protocol Steps

Omitted reagent	Replaced reagent	Added reagent	Number of isolations	Number of successful isolations	% of successful isolations
Creatine	Collagenase IV → collagenase II		14	9	64
Taurine	Collagenase IV → collagenase II		7	3	43
Blebbistatine	Collagenase IV → collagenase II		6	3	50
Creatine, taurine	Collagenase IV → collagenase II		5	2	40
Creatine, blebbistatine	Collagenase IV → collagenase II		6	3	50
Taurine, blebbistatine	Collagenase IV → collagenase II	O_2	5	4	80
Blebbistatine	Collagenase IV → collagenase II	O_2	11	10	91
Blebbistatine, FBS	Collagenase IV → collagenase II	O_2	17	15	88
Blebbistatine, FBS, calcium reintroduction	Collagenase IV → collagenase II	O_2, Kraftbrühe solution	7	7	100

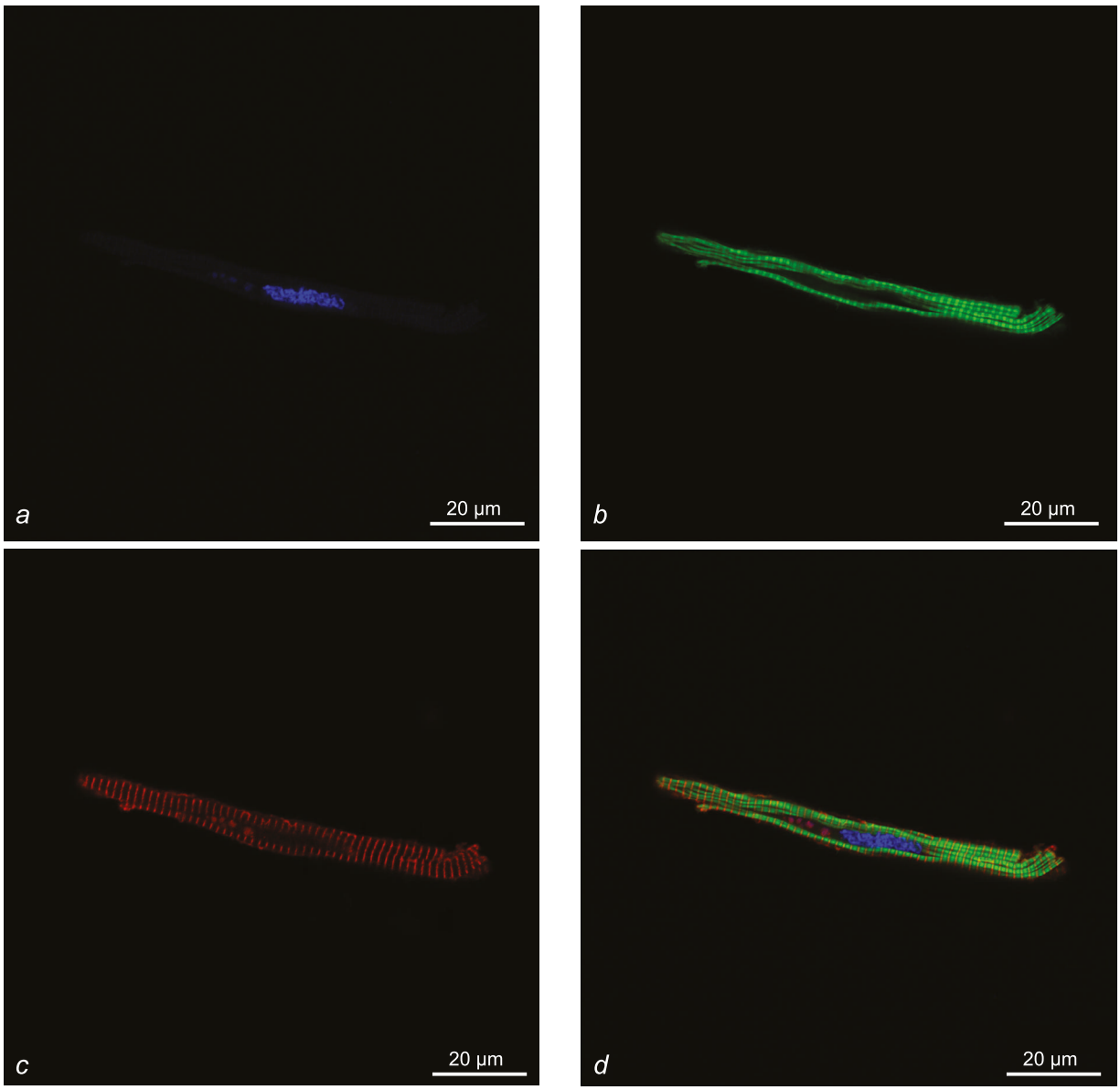

Fig. 3. Mature cardiomyocytes isolated from a biopsy specimen of human right atrial appendage. Confocal microscopy. *a*) Nucleus (DAPI, blue color); *b*) cytoskeletal microfilament, contractile element of muscles (F-actin, green color); *c*) actin-binding protein (α-actinin, red color); *d*) overlay images.

provides increased efficacy with minimal direct impact on cardiac electrophysiology [25]. The absence of each of the three reagents or two of them simultaneously impaired cardiomyocyte survival.

To further increase the percentage of successful isolations with a live cell yield, calcium-free solution during 30-min "rest" (cell membrane repair) was replaced with a cold Kraftbrühe solution (in mM: 50 potassium glutamate, 20 HEPES, 20 taurine, 3 $MgSO_4$, 30 KCl, 0.5 EGTA, 30 KH_2PO_4, and 10 glucose) [26] saturated with oxygen (Table 2).

It is believed that the saturation of solutions with oxygen during cell isolation is necessary for their survival [27], but this is not the most important factor in the isolation of cardiomyocytes [19]. Relying on our own experience, we believe that oxygen can increase the yield of live cardiomyocytes even in the absence of some reagents responsible for energy metabolism.

Structure of isolated cardiomyocytes. The structure of mature isolated atrial cardiomyocytes was studied by immunocytochemistry on a confocal scanning microscope. The nucleus, cytoskeleton, and striated structure typical of cardiomyocytes were not damaged in the isolated cells (Fig. 3).

Electrophysiology of mature cardiomyocytes. All isolated mature cells selected for current recording by the patch-clamp method, had a rod-like shape and striated structure.

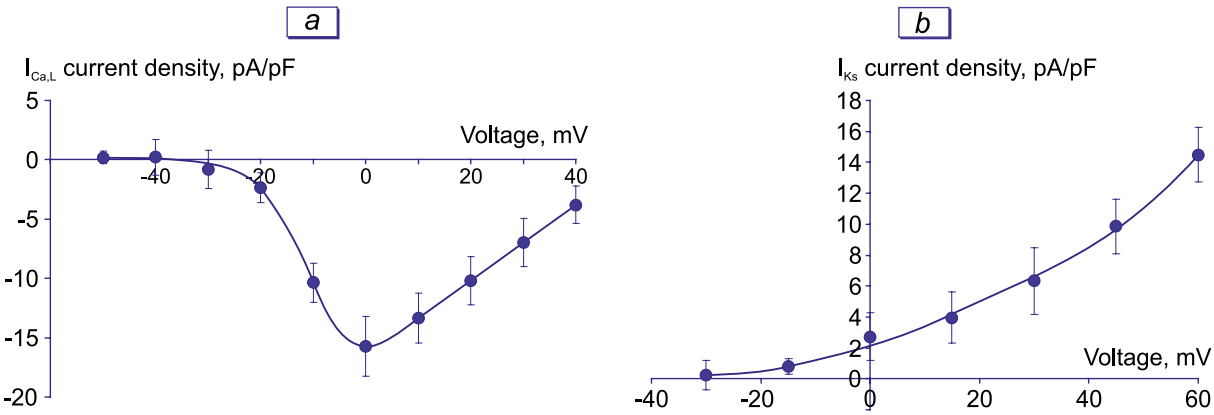

Fig. 4. Current-voltage curve of L-type calcium channels $I_{Ca,L}$ (*n*=4; *a*) and slow potassium channels I_{Ks} (*n*=6; *b*).

The current-voltage characteristics of currents in voltage-gated channels responsible for action potential formation were studied: L-type calcium channels $I_{Ca,L}$ and slow potassium channels I_{Ks} (Fig. 4). The current-voltage ratio I-V showed that the peak density of calcium currents was at 0 mV and the mean maximum peak of $I_{Ca,L}$ normalized to the cell capacitance was -15.74±2.53 pA/pF (*n*=4) (Fig. 4, *a*). For I_{Ks}, the mean current amplitude normalized to the cell capacitance was 14.99±1.79 pA/pF (*n*=6) (Fig. 4, *b*).

Thus, the composition of the buffer solution for isolation of healthy human cardiomyocytes from the heart biopsy specimens should include creatine, blebbistatin, and taurine. The optimized protocol allows more efficient use of biopsy specimens and increases the yield of live cells.

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Conflict of interest. The authors declare no conflict of interest.

REFERENCES

1. Ackerman MJ, Mohler PJ. Defining a new paradigm for human arrhythmia syndromes: phenotypic manifestations of gene mutations in ion channel- and transporter-associated proteins. Circ. Res. 2010;107(4):457-465. doi: 10.1161/CIRCRESAHA.110.224592
2. Blechschmidt S, Haufe V, Benndorf K, Zimmer T. Voltage-gated Na+ channel transcript patterns in the mammalian heart are species-dependent. Prog. Biophys. Mol. Biol. 2008;98(2-3):309-318. doi: 10.1016/j.pbiomolbio.2009.01.009
3. Zicha S, Moss I, Allen B, Varro A, Papp J, Dumaine R, Antzelevich C, Nattel S. Molecular basis of species-specific expression of repolarizing K^+ currents in the heart. Am. J. Physiol. Heart Circ. Physiol. 2003;285(4):H1641-H1649. doi: 10.1152/ajpheart.00346.2003
4. Oh JG, Kho C, Hajjar RJ, Ishikawa K. Experimental models of cardiac physiology and pathology. Heart Fail. Rev. 2019;24(4):601-615. doi: 10.1007/s10741-019-09769-2
5. Protze SI, Lee JH, Keller GM. Human pluripotent stem cell-derived cardiovascular cells: from developmental biology to therapeutic applications. Cell Stem Cell. 2019;25(3):311-327. doi: 10.1016/j.stem.2019.07.010
6. Andrysiak K, Stępniewski J, Dulak J. Human-induced pluripotent stem cell-derived cardiomyocytes, 3D cardiac structures, and heart-on-a-chip as tools for drug research. Pflugers Arch. 2021;473(7):1061-1085. doi: 10.1007/s00424-021-02536-z
7. Ronaldson-Bouchard K, Ma SP, Yeager K, Chen T, Song L, Sirabella D, Morikawa K, Teles D, Yazawa M, Vunjak-Novakovic G. Advanced maturation of human cardiac tissue grown from pluripotent stem cells. Nature. 2018;556:239-243. doi: 10.1038/s41586-018-0016-3
8. Karbassi E, Fenix A, Marchiano S, Muraoka N, Nakamura K, Yang X, Murry CE. Cardiomyocyte maturation: advances in knowledge and implications for regenerative medicine. Nat. Rev. Cardiol. 2020;17(6):341-359. doi: 10.1038/s41569-019-0331-x
9. Slotvitsky MM, Tsvelaya VA, Frolova SR, Dement'eva EV, Agladze KI. The study of the functionality of cardiomyocytes obtained from induced pluripotent stem cells for the modeling of cardiac arrhythmias based on long QT syndrome. Vavilov. Zh. Genet. Selekts. 2018;22(2):187-195. Ruddisn. doi: 10.18699/VJ18.346
10. Jost N, Virág L, Bitay M, Takács J, Lengyel C, Biliczki P, Nagy Z, Bogáts G, Lathrop DA, Papp JG, Varró A. Restricting excessive cardiac action potential and QT prolongation: a vital role for IKs in human ventricular muscle. Circulation. 2005;112(10):1392-1399. doi: 10.1161/CIRCULATIONAHA.105.550111
11. O'Hara T, Virág L, Varró A, Rudy Y. Simulation of the undiseased human cardiac ventricular action potential: model formulation and experimental validation. PLoS Comput. Biol. 2011;7(5):e1002061. doi: 10.1371/journal.pcbi.1002061
12. ten Tusscher KH, Noble D, Noble PJ, Panfilov AV. A model for human ventricular tissue. Am. J. Physiol. Heart

Circ. Physiol. 2004;286(4):H1573-H1589. doi: 10.1152/ajpheart.00794.2003
13. Peeters GA, Sanguinetti MC, Eki Y, Konarzewska H, Renlund DG, Karwande SV, Barry WH. Method for isolation of human ventricular myocytes from single endocardial and epicardial biopsies. Am. J. Physiol. 1995;268(N 4, Pt 2):H1757-H1764. doi: 10.1152/ajpheart.1995.268.4.H1757
14. Bird SD, Doevendans PA, van Rooijen MA, Brutel de la Riviere A, Hassink RJ, Passier R, Mummery CL. The human adult cardiomyocyte phenotype. Cardiovasc. Res. 2003;58(2):423-434. doi: 10.1016/s0008-6363(03)00253-0
15. Bistola V, Nikolopoulou M, Derventzi A, Kataki A, Sfyras N, Nikou N, Toutouza M, Toutouzas P, Stefanadis C, Konstadoulakis MM. Long-term primary cultures of human adult atrial cardiac myocytes: cell viability, structural properties and BNP secretion in vitro. Int. J. Cardiol. 2008;131(1):113-122. doi: 10.1016/j.ijcard.2007.10.058
16. Voigt N, Pearman CM, Dobrev D, Dibb KM. Methods for isolating atrial cells from large mammals and humans. J. Mol. Cell. Cardiol. 2015;86:187-198. doi: 10.1016/j.yjmcc.2015.07.006
17. Zhou B, Shi X, Tang X, Zhao Q, Wang L, Yao F, Hou Y, Wang X, Feng W, Wang L, Sun X, Wang L, Hu S. Functional isolation, culture and cryopreservation of adult human primary cardiomyocytes. Signal Transduct. Target Ther. 2022;7(1):254. doi: 10.1038/s41392-022-01044-5
18. Shumakov DV, Agladze KI, Zybin DI, Popov MA, Frolova SR kyzy, Romanova SG. Patent RU No. 2749986. Method for isolation of cardiomyocytes from human heart tissue. Bull. No. 18. Published June 21, 2021.
19. Shumakov DV, Agladze KI, Zybin DI, Agafonov EG, Popov MA, Romanova SG, Frolova ShR, Slotvitsky MM, Berezhnoy AK, Tsvelaya VA. Status of INA in atrial cardiomyocytes after administration of cardioplegia. Klin. Eksper. Khir. 2022;10(2):26-32. Russian. doi: 10.33029/2308-1198-2022-10-2-26-32
20. Frolova SR, Gorbunov VS, Shubina NS, Perepukhov AM, Romanova SG, Agladze KI. Stilbene derivative as a photosensitive compound to control the excitability of neonatal rat cardiomyocytes. Biosci. Rep. 2019;39(1):SR20181849. doi: 10.1042/BSR20181849
21. Podgurskaya AD, Slotvitsky MM, Tsvelaya VA, Frolova SR, Romanova SG, Balashov VA, Agladze KI. Cyclophosphamide arrhythmogenicity testing using human-induced pluripotent stem cell-derived cardiomyocytes. Sci. Rep. 2021;11(1):2336. doi: 10.1038/s41598-020-79085-5
22. Del Franco A, Ambrosio G, Baroncelli L, Pizzorusso T, Barison A, Olivotto I, Recchia FA, Lombardi CM, Metra M, Ferrari Chen YF, Passino C, Emdin M, Vergaro G. Creatine deficiency and heart failure. Heart Fail. Rev. 2022;27(5):1605-1616. doi: 10.1007/s10741-021-10173-y
23. Balestrino M. Role of creatine in the heart: health and disease. Nutrients. 2021;13(4):1215. doi: 10.3390/nu13041215
24. Dou Y, Arlock P, Arner A. Blebbistatin specifically inhibits actin-myosin interaction in mouse cardiac muscle. Am. J. Physiol. Cell Physiol. 2007;293(3):C1148-C1153. doi: 10.1152/ajpcell.00551.2006
25. Swift LM, Kay MW, Ripplinger CM, Posnack NG. Stop the beat to see the rhythm: excitation-contraction uncoupling in cardiac research. Am. J. Physiol. Heart Circ. Physiol. 2021;321(6):H1005-H1013. doi: 10.1152/ajpheart.00477.2021
26. Kamkin AG, Kamkina OV, Shim AL, Bilichenko A, Mitrokhin VM, Kazansky VE, Filatova TS, Abramochkin DV, Mladenov MI. The role of activation of two different sGC binding sites by NO-dependent and NO-independent mechanisms in the regulation of SACs in rat ventricular cardiomyocytes. Physiol. Rep. 2022;10(7):e15246. doi: 10.14814/phy2.15246
27. Cai B, Mu X, Gong D, Jiang S, Li J, Meng Q, Bai Y, Liu Y, Wang X, Tan X, Yang B, Lu Y. Difference of sodium currents between pediatric and adult human atrial myocytes: evidence for developmental changes of sodium channels. Int. J. Biol. Sci. 2011;7(6):708-714. doi: 10.7150/ijbs.7.708