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An Efficient and Convenient Method for Isolation and Culturing of Neonatal Rat Cardiomyocytes

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For isolation of neonatal rat cardiomyocytes (NRCM) the ventricular muscles of neonatal rats were treated with different digestive solutions: 0.06% trypsin (method I), 0.08% collagenase II (method II), 0.06% trypsin and 0.08% collagenase II for stepwise digestion (methods III and IV). After enzymatic dissociation of the tissue, the complete medium was added to stop this process. The cells suspensions obtained by methods I-III were collected and centrifuged. In contrast, the novel and improved method IV did not use centrifugation. Instead, various methods of adhesion were employed to separate non-myocardial cells. The isolation methods were compared by the quantity, survival rate, morphology, spontaneous pulsation rate, purity, and vitality of NRCM. These assessments showed that isolation method IV is a simple, efficient, and convenient way to obtain NRCM for culturing.

Key Words: *neonatal rat cardiomyocytes (NRCM); cell isolation; stepwise tissue digestion; hypoxia/reoxygenation; Curcuma aromatica extract*

As cardiac diseases are among the major causes of death worldwide, much efforts are focused on the development of pharmacological models of heart diseases [5]. Cultured cardiomyocytes are widely used for studying the morphological, electrophysiological, and biomechanical parameters of these cells and the process of myofibrillogenesis [1,3].

Neonatal rat cardiomyocytes (NRCM) have some advantages over cells from adult rats: they are convenient, low-cost, more stable, and less sensitive to changes in Ca^{2+} concentration in the medium. Moreover, NRCM typically start spontaneously beating after 20-h culturing. The contractile profile of NRCM during hypoxia/reoxygenation are comparable with that of hearts exposed to ischemia/reperfusion *in situ* [7].

Isolation of NRCM for studying the cardiovascular diseases and testing cardiac drugs is the key step. To this end, classical or modified protocols of NRCM isolation proposed by Harary and Farley who first reported successful isolation and culturing of rodent cardiomyocytes in 1963. During the past years, most modifications mainly consisted in selection of digestive enzymes (single enzyme or mixed enzymes) including concentration, proportion, and duration of tissue digestion, as well as choosing serum concentration and growth supplement for cell attachment [8]. Trypsin (0.05-0.25%) and collagenase II (0.05-0.1%) are commonly used as biological enzymes.

NRCM is usually isolated by single-step treatment with single enzyme or mixed enzymes, rather than by stepwise treatment. Whatever method is used, the cell suspension is centrifuged after tissue digestion [6]. In this paper, 4 methods of NRCM isolation were tested in parallel: 0.06% trypsin digestion with centrifugation (I); 0.08% collagenase II digestion with centrifugation (II); stepwise digestion with 0.06% trypsin and 0.08% collagenase II followed by centrifugation (III); stepwise digestion with 0.06% trypsin and 0.08% collagenase II without centrifugation (IV).

MATERIALS AND METHODS

Neonatal Sprague-Dawley (SD) rats (age 2-3 days) were purchased from Laboratory Animal Center of the

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Guangxi Medical University. The experiments were approved by the Committee on Animal Care and Use of the Guangxi Medical University) and were performed in accordance with international principles for humane treatment of animals.

The chest was opened along the sternum, the whole heart was excised and immediately transferred to a sterile Petri dish with pre-cooled PBS containing (in mmol/liter): 137 NaCl, 2.7 KC1, 4.3 Na_2HPO_4, and 14 KH_2PO_4 (pH 7.2-7.4). Non-ventricular tissues (auricles and atria) were carefully removed, the ventricles were washed 3 times with fresh chilled PBS, cut into small pieces (1-3 mm^3), and placed in a sterile penicillin bottle with 1 ml chilled PBS. Then, the fragments were transferred to a sterile 15 ml centrifuge tube with 6 ml 0.06% trypsin. The tissue fragments were intermittently stirred for 2 min and then allowed to stay for 1 min at 37°C. This step was repeated for 3 times, and then the supernatant containing non-cardiac cells was discarded. The precipitate was divided into 4 groups (for 4 isolation protocols). The precipitates were treated with 0.06% trypsin or 0.08% collagenase II or successively by these enzymes to collect cell suspension containing no tissue fragments.

First stage. After the tube content became soft and fluffy (15 min), the cell supernatant was carefully collected in another 15 ml centrifuge tube, then equal volume of complete medium (high glucose DMEM supplemented with 20% inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin) was added to stop digestion, and the collected supernatant was preserved at 37°C. At this stage, the digestions were 0.06% trypsin (I), 0.08% collagenase II (II), and 0.06% trypsin (III and IV).

Second stage. The rest of tissue pieces were then resuspended in fresh digestive solution for 25-30 min until all the tissue disappeared. Then, equal volume of complete medium was added to stop digestion. The digestions were 0.06% trypsin (I), 0.08% collagenase II (II), and 0.08% collagenase II (III and IV).

The cell suspension from the two steps were combined, then passed through a Falcon 100 μm Cell Strainer (BD) to prepare cell suspension containing no tissue fragments. In groups I-III, the cell suspensions were centrifuged at 400*g* for 10 min. In group IV, the cell suspension was not centrifuged.

To obtain myocytes, the dissociated cells obtained by the above 4 methods were cultured in a humidified incubator (5% CO_2, 95% O_2) at 37°C for 2 h to allow differential attachment of non-myocardial cells. Non-attached NRCM were collected, resuspended in high-glucose complete medium containing 100 μM 5-bromo-2'-deoxyuridine, and seeded into fresh culture dishes, 6-well, or 96-well plates. The medium was changed after 48 h and then renewed every day. All the cell isolation and culturing steps were operated under aseptic conditions.

H9c2 cells (obtained from the Cell Bank of Chinese Academy of Science (Beijing, China) were cultured at 37°C in a humidified incubator (5%CO_2 and 95% O_2), culture medium was changed every day. H9c2 cells in logarithmic growth phase were seeded at a density of 6×10^3 to wells of a 96-well plates containing 100 μl medium, and incubated in a nutrient-rich medium at 37°C in humidified atmosphere with 5% CO_2 for 24 h.

Trypan blue exclusion test was used to assess the separation quantity and survival rate of NRCM after the cells were incubated for 2 h. To this end, 100 μl NRCM suspension from each group was transferred into a 1.5 ml sterile tube containing 900 μl 0.04% trypan blue solution and incubated for 3 min (dead cells turned blue). The numbers of isolated and viable NRCM were counted using a hemocytometer. Each experiment was repeated 3 times, and the results were expressed as $M\pm SD$.

Morphological changes and growth state of each group of NRCM were consecutively observed on days 2-15, and the frequency of spontaneous contractions in each group of NRCM was recorded under an inverted microscope in 5 randomly chosen cell clusters and expressed in $M\pm SD$.

To demonstrate the cells in cultures were NRCM and to verify their purity, the cells were assayed by confocal immunofluorescence. To this end, the cells were washed with PBS and fixed in 4% paraformaldehyde for 30 min, then washed with PBS and permeabilized for 15 min with 0.2% Triton X-100. After washout with PBS, the cells were incubated with Rabbit Anti-Sarcomeric Alpha Actinin (α-SA, 1:50, Abcam**)** overnight at 4°C. Then, NRCM were washed 3 times with PBS, incubated with FITC-labeled goat anti-rabbit secondary antibody (1:200, Sigma) for 60 min, washed with PBS, and then incubated with DAPI for 10 min at room temperature (all washout procedures were repeated 3 times, 3 min each). The cells were examined under a Nikon A1 confocal microscope.

MTT assay was used to evaluate the quality of NRCM isolation. In group IV, the cytotoxicity of the isolation procedure was assayed, and in 4 groups and in H9c2 cell cultures, cell viability was assessed.

To test the cytotoxicity, 100 μl logarithmic growth phase NRCM isolated by method IV were cultured in 96-well plates for 24 h, then the liquid was removed and replaced with high-glucose DMEM containing *C. aromatica* extract [4] (70% hydroalcoholic *C. aromatica* extract was dissolved in DMSO; the final concentration of DMSO in the medium did not exceed 0.1%, the test concentrations were 2000, 1000, 500, 250, 125, 62.5, 31.25 μg/ml) for 12 h under normoxic conditions in 37°C. The culture medium was removed,

20 μl MTT (0.5 mg/ml) was added, and the cells were incubated at 37°C for 4 h avoiding light. The medium with MTT was removed and 150 μl DMSO was added to the wells. The optical density (OD) was measured and the cell vitality was calculated. From the minimum cytotoxic concentration, we can determine the concentrations of *C. aromatica* extracts in the following experiments.

The NRCM hypoxia/reoxygenation model *in vitro* was established as described elsewhere [2]. Each well was repeated for 3 times. The control cell was cultured with high-glucose DMEM without FBS under normoxic conditions for equivalent duration. After exposure to hypoxia/reoxygenation, the cells were incubated with 0.5 mg/ml MTT at 37°C for 4 h. The supernatant was removed and 150 μl DMSO was added to the wells.

In parallel, H9c2 cells were pre-treated with *C. aromatica* extracts and exposed to hypoxia/reoxygenation as above; OD was measured at 570 nm using a Microplate Reader (Thermo Scientific). The results were expressed as $M \pm SD$. Formula used to calculate cell vitality rate was:

$$\text{Cell vitality rate} = (OD_{extract} - OD_{medium})/(OD_{blank} - OD_{medium}) \times 100\%.$$

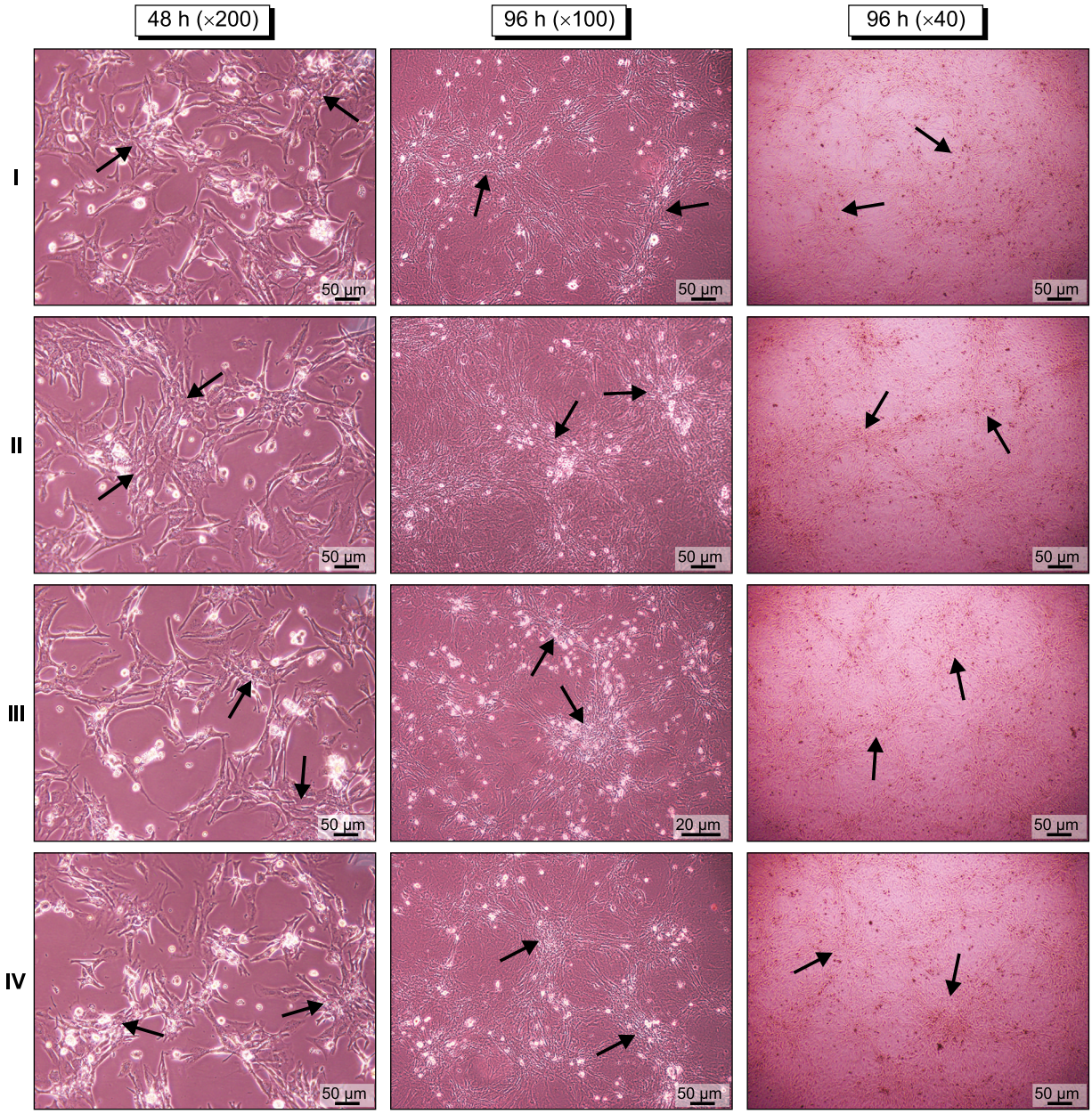

Fig. 1. Morphology of NRCM at different terms of culturing. In 48 h in culture, contacting pseudopodia were revealed (arrow). In 96 h, inter-connected dense network formed by radially arranged cell clusters was seen (arrow).

The results of confocal immunofluorescence microscopy were analyzed by Image-Pro Plus 6.0 software. The data were expressed as $M \pm SD$ and analyzed using SPSS Statistics software (IBM). Statistical significance was analyzed using one-way ANOVA; the differences were significant at $p<0.05$.

RESULTS

There were no significant differences between the groups in the total number of isolated cells. In terms of cell survival rate, method IV yielded significantly higher number of viable cells that method I ($p<0.05$).

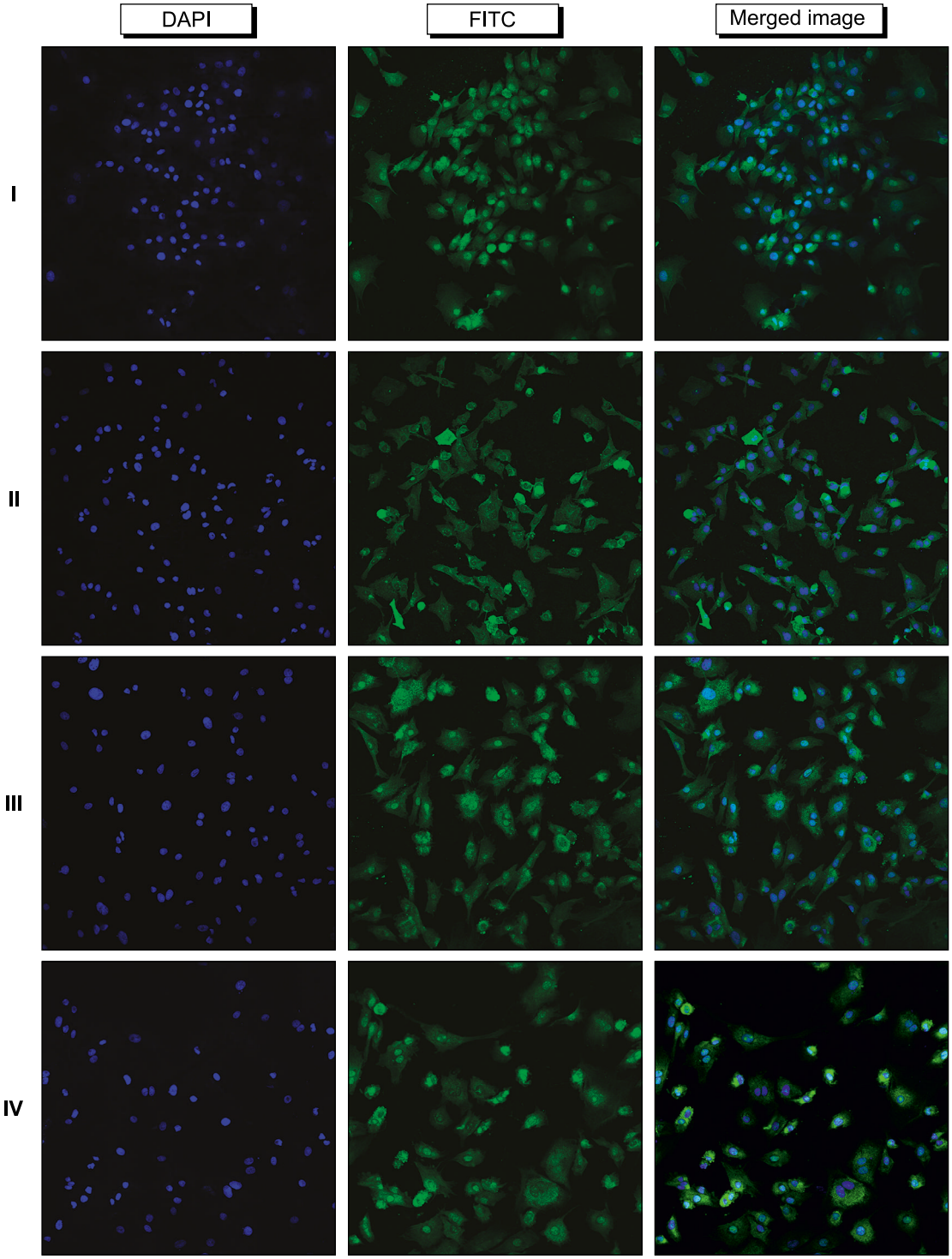

Fig. 2. Purity of NRCM isolation by four methods. The nuclei were stained with DAPI (blue), α-SA staining with FITC (green), ×200.

No significant differences in the cell survival rate between methods II, III, and IV were revealed.

Adherent NRCM were found after 12 h in culture, spontaneous contractions of NRCM were observed after 24 h, and then, the cells extended polygonal pseudopodia. The crosslinked NRCM were found in 48 h, the mutual contact pseudopodia formed NRCM clusters in 72 h. In 96 h, inter-connected dense network formed by radially arranged cell clusters demonstrated island-like foci of beating (Fig. 1). Actively contracting cells were continuously recorded from day 3 to day 15 in culture, and the synchronic rates were at 60-100 bpm (Table 1). There were no significant differences in cell morphology and their contractile activity between NRCM isolated by method IV and other 3 methods.

Immunofluorescence staining revealed NRCM positively stained for α-SA in cultures isolated by all 4 methods (Fig. 2). Although non-cardiomyocyte cells (*e.g.* fibroblasts) could also be stained, their nuclei were not surrounded by α-SA. The purity of methods I-IV was 85.70, 90.09, 97.39, and 97.70%, respectively (n=3).

C. aromatica extract in concentrations <125 μg/ml was considered as non-cytotoxic (Fig. 3, *a*). Therefore, we used the concentrations of *C. aromatica* extracts (125, 62.5, 31.25, 15.625, 7.8125, and 0 μg/ml) for the subsequent viability experiment on H9c2 and NRCMs in parallel.

The viability of NRCMs and H9c2 cells exposed to hypoxia/reoxygenation was consistent with the data obtained *in vivo* and showed good dose-dependent relationship (Fig. 3, *b*) [7]. However, the response of NRCM to the same *C. aromatica* extract doses was more pronounced than that of H9c2 cells. This meant that all 4 methods of NRCM isolation can be used for cardiovascular disease modeling and drug research. At the same time, method IV is simple and is characterized by high survival rate, high purify.

Thus, there were no significantly differences in the NRCM yield, morphology, spontaneous pulsation rates, and MTT test results between the four methods. The survival rates and purities of cultured obtained by methods II-IV were significantly higher than those

TABLE 1. Spontaneous Contractile Activity of NRCM (bpm) at Different Terms in Culture (n=5)

Day in culture	Method of isolation			
	I	II	III	IV
2	37±7	40±8	38±6	37±7
3	80±8	84±7	82±7	84±8
4	81±4	82±4	83±2	85±4
5	84±4	83±3	86±2	85±4
6	84±4	85±2	85±4	87±2
7	87±3	86±3	87±2	86±4
8	86±2	86±3	88±3	86±2
9	87±3	86±3	87±3	85±2
10	86±4	85±4	86±3	88±3
11	87±3	85±3	86±5	86±4
12	80±4	79±3	80±3	80±4
13	79±7	84±3	80±4	81±5
14	76±7	73±4	76±4	77±1
15	76±6	75±7	76±5	74±4

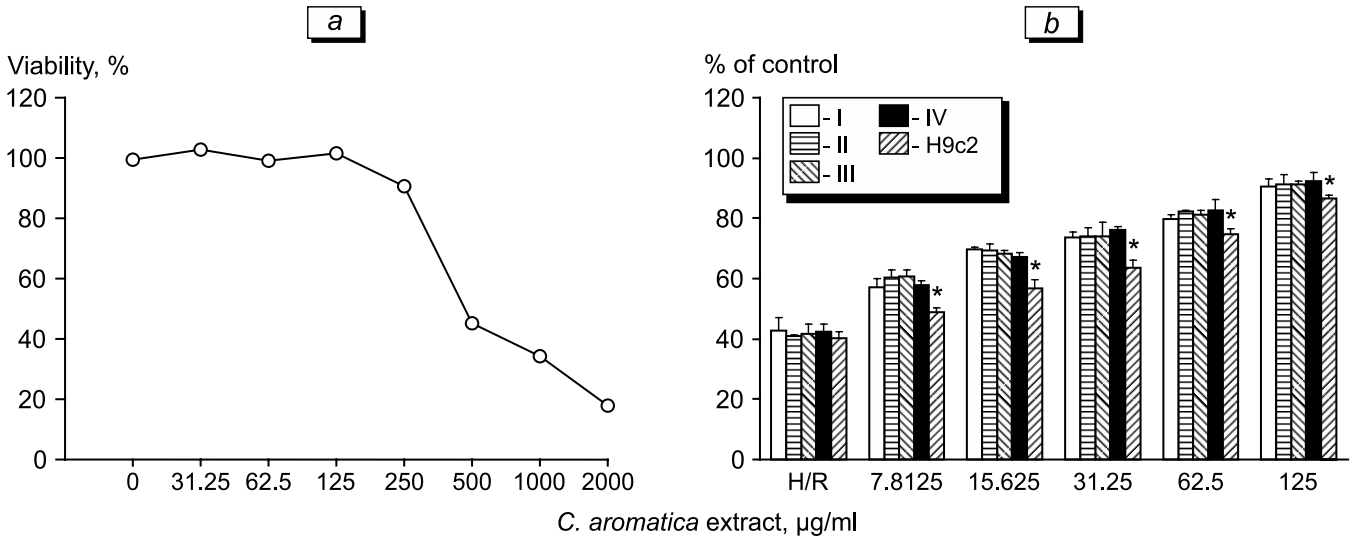

Fig. 3. Evaluation of cytotoxicity and hypoxia/reoxygenation (H/R) tests. *a*) Cytotoxicity of method IV of NRCM isolation. Control cell cultured in high-glucose DMEM without FBS under normoxic conditions served as the control (100% viability). *b*) Viability of NRCM and reference H9c2 cells pre-treated with different concentrations of *C. aromatica* extract. *p<0.05 in comparison with H9c2 cells.

of method I (p<0.05). Methods III and IV employed stepwise digestion, but method IV did not use centrifugation. So, method IV could be considered as a simple, efficient, and convenient new method to isolate NRCM.

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