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PRIMARY STUDY FOR POSSIBLE TRANSDIFFERENTIATION EFFECTS OF SEVERAL VIETNAMESE PLANT EXTRACTS ON ISOLATED RAT NEONATAL CARDIOMYOCYTES

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Abstract. Cardiovascular diseases have been accounted for the highest mortality rate worldwide. Sinoatrial node (SAN) dysfunction causes many symptoms from syncope to cardiac arrest, which usually happened in elderly people. Recently, there are many strategies that were developed to support the patient. Electrical pacemaker was known as the standard device that was implanted to induce the electrical pulse, with some limitation of the battery and the lead. Transdifferentiation of neonatal cardiomyocytes into pacemaker cells under natural compounds'treatment was recognized as a novel design. Following this direction, we studied the neonatal cardiomyocyte transdifferentiating effects of garlic and green tea extracts. The cardiomyocytes, isolated from neonatal rats, were incubated for 18 hours and exposed to extracts for 24 hours. The garlic and green tea crude extracts or fractions at lower than 100 µg/mL were not toxic for the cells. Among the others, the garlic ethyl acetate fraction seemed to enhance the beating rate of induced cardiomyocytes at 71.33 ± 5.21 beats per minute. This fraction and the ethanol extract from green tea were also able to induce the expression of hyperpolarization-activated cyclic nucleotide-gated channel 4 (HCN4), the important factor for automatic beating of SAN, but insignificantly ($P > 0.05$). In conclusion, neonatal cardiomyocytes were isolated and suitable for use as a model for screening of pacemaker cell transduction effects of potential candidates. From this study, the garlic ethyl acetate fraction presented as the most promising nominee for further neonatal cardiomyocyte transdifferentiating effects.

Keywords: cardiomyocytes, HCN4, pacemaker cell, sinoatrialnode, transdifferentiation.

Classification numbers: 1.2.1, 1.2.2.

1. INTRODUCTION

In a healthy heart, sinoatrial node (SAN) cells are the primary or dominant pacemaker cells accounting for a small number at right atria that produce electrical impulse in heart [1] Besides,

cardiomyocytes or muscle cells account for 35 % of total. However, they are non-pacemaker cells but accept the electrical signal from pacemaker cell to induce contraction [2]. The electrical signal from SAN is quickly and spontaneously spread to the other parts of conduction system such as atrioventricular (AV) node, *His* bundle, bundle branches, and purkinje fibers, resulting in contraction of ventricle to distribute blood into the body [3]. One of the most important ion channels in SAN cell is known as hyperpolarization-activated cyclic nucleotide-gated 4 (HCN4) channel inducing funny current (I_f) that is activated at hyperpolarization stage. This ion channel serves as a regulator of the repetition and the automaticity of action potential in the heart [4]. HCN4 is accounted for automatic beating of SAN cells and the highest expressed isomers in those cells [5,6]. As recognized, HCN4 high expression is among unique characters of SAN cells. Therefore, HCN4 is a gene required for development of pacemaker cells in heart and thus considered as very important criteria for distinguishing SAN cells from other pacemakers and cardiomyocytes.

As reported, there are 0.17 % of the elderly (more than 65 year old) that have SAN dysfunction which also occur in babies by hereditary with about 600,000 pacemakers that are implanted every year worldwide [1]. The first electrical device was developed and tested in 1958, which contained a pulse source and lead system and was implanted into the body. Until now, electrical pacemakers have been optimized for size, functionality and longevity as well [7]. However, they still have adverse impacts such as failure of function or infections of generators or leads. Thus, there is an increasing need for new, safer and more effective replacement of mechanical pacemakers.

In another effort, biological pacemakers using gene or cell therapy are alternative to previous electronic devices. In 2013, the different way known as somatic reprograming that was overexpression of the human embryonic transcription factor TBX18 (T-Box Transcription Factor 18) to convert cardiomyocytes into induced SAN (iSAN) cells presenting pacemaker activity such as I_f current [3]. In another report, the recombination of TBX18 gene and adipose-derived stem cells (ADSCs) was co-cultured with neonatal rat ventricular cardiomyocytes (NRVMs), which generated pacemaker-like cells with the high level expression of HCN4 [8]. However, adenoviruses carrying TBX18 also contained noncoding gene that inhibited cellular defense and faced with limitation in immune system [9]. From another study, Saito reported that overexpressed HCN4-in mouse embryonic stem cells (mESC) presented genes required for impulse conduction, showing rapid spontaneous beating, responding to an I_f inhibitor and beta-adrenergic receptor agonist, and having pacing ability in an *in vitro* co-culture system with other excitable cells [10]. Based on this research, author confirmed that HCN4-overexpressing mESC-derived cardiomyocytes (mESC-CMs) had significantly increased beating rate (87.4 ± 11.9 beats/min) compared with that of the control (43.1 ± 4.8 beats/min) ($P < 0.0001$).

Herbs have been used in traditional medicine for a long time to treat a category of diseases. The previous studies suggested that approximately one-fourth of the commercially available plant-derived drugs are used in traditional medicine, including those used in the treatment of cardiovascular diseases. There are 10 plant species which have been reported as the most popularly used for heart healthy supporting, including garlic (*Allium sativum*) and green tea (*Camellia sinensis*) [11]. In Viet Nam, those plants are also well known as the popular traditional herbs used for strengthening cardiovascular system. In previous reports, garlic extract was recognized that played function in protecting blood vessels for cardiac hypertrophy. Meanwhile, epigallocatechin-3 gallate (EGCG) from green tea was found to significantly reduce the expression of a major cardiac gap junction (Cx43) in ventricular cardiomyocytes from neonatal rats [12, 13]. Therefore, those plants are studied in the present work for their possible

effects to transdifferentiate cardiomyocytes into iSAN in terms of beating rate inducing and HCN4 gene expression in this research.

2. MATERIAL AND METHODS

2.1. Plant extraction

The garlic and green tea leaves were collected at the end of March 2019 in Thai Nguyen city, Viet Nam. The plant was identified and voucher specimens were saved at the Bioassay Laboratory, Institute of Biotechnology, Vietnam Academy of Science and Technology (VAST). The samples were then washed away from dust under tap water before leaving to dry in the shade at room temperature (RT). After several days, the materials were dried in an oven at about 50 °C until well-dried. The well-dried samples were ground into powder which was then soaked in 96 % ethanol at RT for four times and filtered. The filtrates were removed of solvent thoroughly under reduced pressure to obtain crude ethanolic extracts. This crude extracts were then fractionated using flash column chromatography with *n*-hexane, ethyl acetate, dichloromethane, respectively. The crude extract and fractions were kept at -20 ^{o}C for further experiments.

2.2. Cardiomyocytes isolation

Cardiomyocytes were directly isolated following the method from [14] with 1- to 2-day-old Sprague-Dawley rat. Ventricle hearts were removed and washed with cold Phosphate buffered saline (PBS) then isolated with collagenase II 0.372 IU/mL until obtaining a mixture of various cell types like fibroblast, cardiomyocytes, Purkinje fibers. For removal of fibroblasts, the cell suspension was next plated into the culture flask and incubated for 1 hour, at 37 °C. After that, the suspended cells were counted and mono-layer-plated at about 125,000 cells per cm^2 into 96-well plate coated with gelatin or collagen [15]. Cardiomyocytes were grown with DMEM-F12 medium including 1 % penicillin/streptomycin, 10 % Fetal bovine serum, 1 % sodium pyruvate, 1 % D-glucose, 1 % ascorbic acid, 1 % HEPES [15].

2.3. Cytotoxicity MTT assay

The cardiomyocytes treated with samples were incubated in 5 % CO_2, at 37 °C, for 24 hours. Then 20 μL of MTT stock (5 mg/mL) was included in each well which was further kept at 37 °C for 4 hours. After that, the medium was removed and the wells were rinsed with PBS, followed by drying for about 2 hours and adding 200 μL of DMSO to dissolve formazan crystals. The absorbance was measured at 570 nm by the spectrometer [16].

2.4. Inducing beating rate

After incubated with samples for 24 hours as in cytotoxic assay, the cardiomyocytes were washed with PBS three times and maintained in the DMEM-F12 cultured medium for 3 more days. At this day 3, the beating rate of cardiomyocytes was visually observed by camera-anchored microscope (Nikon model DZAPHOT, Camera Canon DS126431, Japan) which recorded the beating during 10 seconds. All the beating rates were presented as beat per minute (bpm) [17].

2.5. Gene expression

After incubated with samples for 24 hours as in cytotoxic assay, the cardiomyocytes were washed with PBS three times and maintained in the DMEM-F12 cultured medium for 5 more days. At the day 5, total cells in all experimented groups were washed with 100 µLof PBS, and then incubated in 350 µL 1% (v/v) β-mercaptoethanol diluted with extraction buffer RLT (Qiagen) for 3 minutes, and collected into each 1.5 ml tubes for RNA extraction and purification. RNAs were further purified according to the manufacturer's protocol by RNeasy Mini Kit (Qiagen). The RNA concentration was measured with NanoDrop™ 1000 (ThermoScientific, USA). Then, complementary DNAs (cDNA) were amplified from the total RNA templates via reverse transcription according to the manufacturer's instructions (a SuperScript® III First-Strand Synthesis System, Invitrogen, Carlsbad, CA, USA). Real-time quantitative PCR was performed by Tagman probes and primers for the expressions of the targeted gene HCN4 (Mm01176086_m1, Thermo Fisher Scientific) and housekeeping gene GAPDH (Mm99999915_g1, Thermo Fisher Scientific) on a Roche LightCycler® 96 System (Swiss). Initial steps of RT-PCR were 2 min at 50 °C for degrading nonspecific products, followed by a 10 min at 95°C. Forty cycles were conducted including a 15 sec melting at 95 °C, then a 1 min of extension at 60 C. All reactions were replicated three times. The expression was evaluated through the relative fold change calculated by $2^{-\Delta\Delta Ct}$ in which ΔΔCt was the difference between ΔCt of sample and ΔCt of untreated control.

2.6. Data analysis

The data was reported as mean ± standard deviation (SD), which were analyzed by the GraphPad Prism 7 software using unpaired *t*-test. The $P < 0.05$ was considered statistical significance.

3. RESULTS AND DISCUSSIONS

3.1. Cardiomyocyte isolation

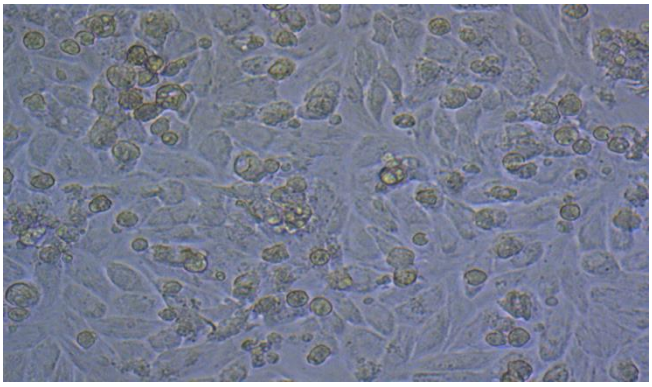

Figure 1. Images of cardiomyocytes isolated from neonatal rat under microscope after 18 hours after plating using Nikon model DZAPHOT, Camera Canon DS126431, Japan (20X magnification).

Cardiomyocytes were isolated from neonatal, and then suspended in culture medium with approximately 5.2×10^5 living cells per 1 ml of medium as mentioned method. The obtained cells were plated for 12-18 hours before further experiments. The isolated cardiomyocytes exhibited healthy state and 80 - 90 % confluence after 12-18 hours. The image of plating cardiomyocytes is presented in Figure 1.

3.2. Cytotoxic results

Every crude extracts and fractions were tested for their cytotoxicity at different concentrations ranging from 4 to 100 µg/mL. All extracts and fractions from garlic and green tea showed not toxic to the isolated cardiomyocytes by inhibiting the cell growth less than 30 % at the highest concentration of 100 µg/mL. The lower concentrations lead to around 90 % cell alive. The percentage of alive cells was determined and shown in the Table 1.

Table 1. Percentage of proliferation of cardiomyocytes after treatment with several extracts from garlic and green tea (n = 3, ± SD).

Conc. (µg/mL)	**Alive cells (%)**							
	n-hexane		Dichloromethane		Ethyl acetate		Crude extract	
	Garlic	Green tea	Garlic	Green tea	Garlic	Green tea	Garlic	Green tea
4	98.1 ± 0.51	94.11 ± 0.47	94.33 ± 0.39	89.97 ± 1.17	90.56 ± 0.99	90.04 ± 0.36	98.67 ± 1.55	94.38 ± 1.24
20	93.6 ± 1.03	81.84 ± 0.81	90.35 ± 0.60	80.42 ± 1.10	78.33 ± 0.50	81.41 ± 0.68	88.26 ± 2.39	85.37 ± 1.57
100	70.28 ± 0.93	78.65 ± 0.98	72.91 ± 0.85	70.13 ± 1.27	70.73 ± 1.49	71.71 ± 1.60	70.28 ± 1.02	72.36 ± 0.68

3.3. Beating rate measurement

Table 2. Beat rate (per minute) of induced cardiomyocytes at the day 3 after the treatment of garlic crude extract and fractions at 4, 20 and 100 µg/mL using camera-anchored microscope (n = 3, ± SD).

Conc. (µg/mL)	**Beat rate (per minute)**				
	Control	n-hexane	Dichloromethane	Ethyl acetate	Crude extract
4	67.67 ± 7.22	60.67 ± 3.48	66.33 ± 6.06	65.67 ± 4.67	64.67 ± 7.69
20	65.33 ± 2.91	61.33 ± 4.48	63.33 ± 8.82	71.33 ± 5.21	68.67 ± 4.26
100	66.67 ± 2.02	60.00 ± 2.89	67.00 ± 5.13	68.33 ± 2.19	66.33 ± 1.86

Table 3. Beat rate (per minute) of induced cardiomyocytes at the day 3 after the treatment of green tea leaf crude extract and fractions at 4, 20 and 100 µg/mL usingcamera-anchored microscope (n = 3, ± SD).

Conc. (µg/mL)	**Beat rate (per minute)**				
	Control	n-hexane	Dichloromethane	Ethyl acetate	Crude extract
4	67.67 ± 7.22	64.33 ± 3.84	62.67 ± 3.84	64.33 ± 4.26	67.00 ± 1.09
20	65.33 ± 2.91	67.00 ± 2.52	58.00 ± 5.51	66.33 ± 6.17	68.30 ± 2.70
100	66.67 ± 2.02	62.67 ± 3.71	53.67 ± 4.48	70.67 ± 7.75	60.00 ± 1.73

After 24 hours of incubation, the beating rate was observed under a 20X magnification microscope. The neonatal cardiomyocytes after treatment with extracts did not show significant increase in the beating rate compared to the negative control sample. The number of beats in the treatment groups is ranging from 60 to 70 bmp. For the garlic extracts, ethyl acetate fraction was able to enhance the beat up to 71.33 ± 5.21 bmp. However, this increasing beat was not

significantly different from that of the control group of which was about 66 beat per minute ($P > 0.05$). Results of 1-minute beats of garlic and green tea extracts at 3 test concentrations of 4, 20, 100 µg/mL are shown in Table 2 and Table 3, respectively.

3.4. Evaluation of HCN4 gene expression

The specific HCN4 gene expression was measured after 5 days of treatment. The garlic crude extract and other n-hexane, dichloromethane fractions did not impact on HCN4 expression in cardiomyocytes comparing with the negative control. However, the result showed the higher expression of HCN4 in induced cadiomyocytes which were treated with ethyl acetate extract (Figure 2).

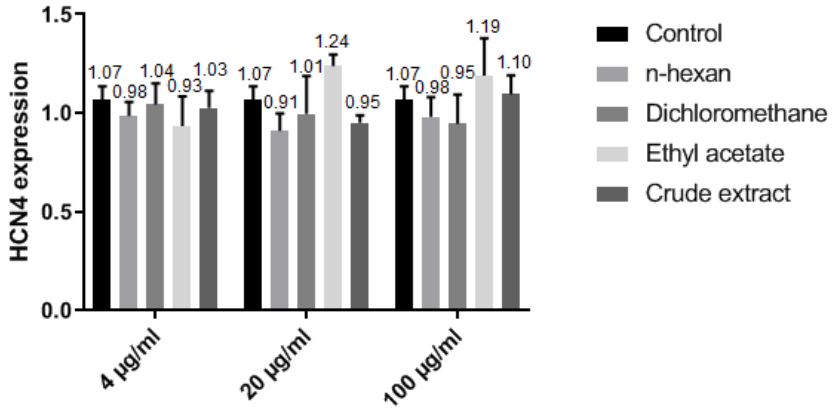

Figure 2. The expression of HCN4 gene in induced neonatal cardiomyocytes after 5 days treatment of garlic crude extract and fractions at several concentrations ranging from 4 to 100 µg/mLusing RT-PCR (n = 3, ± SD).

The isolated cardiomyocytes, which were treated with green tea leaf extract and fractions, exhibited the different levels of HCN4 expression. The target gene seemed to be at the same expressive level among the treated n-hexane, dichloromethane and ethyl acetate groups and the negative control (Figure 3).

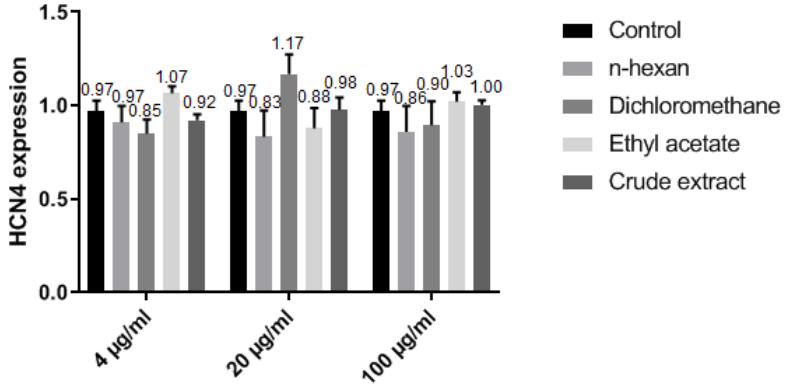

Figure 3. The expression of HCN4 gene in induced neonatal cardiomyocytes after 5 days treatment of green tea leaf crude extract and fractions at several concentrations ranging from 4 to 100 µg/mL using RT-PCR (n = 3, ± SD).

4. DISCUSSION

In this study, we reported our effort to isolate cardiomyocytes from neonatal rat. We tried to screen the transdifferentiation effect of the crude extracts as well as fractions from garlic and green tea leaf which were known as the commonly traditional food supplement for cardiovascular strengthening. As resulted, the isolated cardiomyocytes exhibited healthy condition, round shape and 80 - 90 % confluence after 18 hours plating. The cytotoxicity and the expression of the unique gene were evaluated after treatment with several extracts. There was no extracts from garlic and green tea exhibited toxicity for isolated neonatal cardiomyocytes. However, the results presented that extracts might affect to cell survival through the concentration-dependent manner. Those samples also did not increase the beating rate of neonatal cardiomyocytes significantly comparing with that of control ($P > 0.05$). The ethyl acetate fraction from garlic was the only one that seemed to enhance the beating rate of induced cardiomyocytes from around 71.33 ± 5.21 beats per minute. Besides, HCN4, highly revealed in SAN cell, was a major factor in process of diastolic depolarization and electrical impulse to induce heart beat [18]. In terms of HCN4 gene expression, in this first report, there were two indicated extracts that seemly played function in inducing the gene expressed level. Firstly, the ethyl acetate extract from garlic at concentration of 20 and 100 µg/mL and the aqueous extract from green tea at 20 µg/mL induced HCN4 expressions at 1.19; 1.24 and 1.17 folds change, respectively. However, this increasing was not significantly higher than that of control ($P > 0.05$). Up to now, there has been no reports related to activities of neither garlic nor green tea extracts on neonatal cardiomyocytes transdifferentiation. In other previous reports, the powder from garlic had function in inhibiting several pro-inflammatory cytokines causing heart diseases and the oil extracted from garlic triggered out differentiation in human promyelocytic leukemia cells (HL-60) and the breast cancer cell line MCF7 [19, 20, 21]. Regarding green tea activity, it was shown to contain a great amount of bioactive ingredients, especially flavonoids that was demonstrated as reducing cardiovascular disease [22]. Besides, extract from green tea was figured out the function in differentiation of adipocytes into osteoblast [23]. Thus, our research is the first report on transdifferentiation activities of garlic and green tea extracts on neonatal cardiomyocytes in terms of beating rate measurement and HCN4 gene expression.

5. CONCLUSIONS

The isolated neonatal cardiomyocytes exhibited healthy condition, round shape and 80 – 90 % confluence after 18 hours plating. The extracts from garlic and green tea presented not toxic for isolated neonatal cardiomyocytes at treated concentrations. There was also no extracts from garlic and green tea that was able to induce neonatal cardiomyocytes into iSAN significantly. However, ethyl acetate extract from garlic and aqueous extract from green tea might have impact on the HCN4 gene expression that should be further investigated.

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