УДК 616.12-008.46-036.12-008.853-076.5:615.273.3
https://doi.org/10.20538/1682-0363-2022-2-90-96

Evaluation of the functional state of mitochondria isolated from mononuclear leukocytes by flow cytometry in patients with chronic heart failure receiving ubidecarenone

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ABSTRACT

Aim. To evaluate the functional state of mitochondria isolated from peripheral blood mononuclear leukocytes using flow cytometry in patients with chronic heart failure receiving ubidecarenone (coenzyme Q).

Materials and methods. The study included 53 patients with chronic heart failure who had experienced myocardial infarction. The patients were divided into two groups: group 1 received optimally chosen standard therapy, while group 2 received optimally chosen standard therapy and ubidecarenone ("Kudevite"). The mitochondrial membrane potential was evaluated by flow cytometry using propidium iodide and 3,3′-dihexyloxacarbocyanine iodide (DiOC6(3)). The levels of coenzyme Q were determined using high-performance liquid chromatography with ultraviolet (UV) detection.

Results. A direct correlation was established between the coenzyme Q levels in the blood plasma and the percentage of DiOC6(3)-positive cells ($R = 0.39$; $p < 0.05$) in the patients with chronic heart failure. In group 1, no significant differences in the coenzyme *Q* levels and the percentage of DiOC6(3)-positive and DiOC6(3)-negative cells before and after the therapy were observed. In group 2, a significant increase in the proportion of DiOC6(3)-positive cells and a significant decrease in the percentage of DiOC6(3)-negative cells were revealed.

Conclusion. The increase in the functional activity of mitochondria in the patients with chronic heart failure receiving ubidecarenone was identified. Flow cytometry can be used to evaluate the functional state of mitochondria and observe the efficiency of the selected therapy.

Keywords: mitochondria, chronic heart failure, coenzyme Q, flow cytometry, mitochondrial membrane potential

Conflict of interest. The authors declare the absence of obvious or potential conflict of interest related to the publication of this article.

Source of financing. The authors state that they received no funding for the study.

Conformity with the principles of ethics. All study participants signed an informed consent. The study was approved by the local Ethics Committee at North-Western State Medical University named after I.I. Mechnikov (Protocol No. 12 of 10.12.2014).

For citation: Lobanova O.A., Gaikovaya L.B., Dadali V.A., Ermakov V.A., Kukharchik G.A. Evaluation of the functional state of mitochondria isolated from mononuclear leukocytes by flow cytometry in patients with chronic heart failure receiving ubidecarenone. *Bulletin of Siberian Medicine.* 2022;21(1):90–96. https://doi.org/10.20538/1682-0363-2022-2-90-96.

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Оценка функционального состояния митохондрий мононуклеарных лейкоцитов методом проточной цитометрии у пациентов с хронической сердечной недостаточностью под влиянием убидекаренона

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РЕЗЮМЕ

Цель – оценить функциональное состояние митохондрий мононуклеарных лейкоцитов периферической крови с применением метода проточной цитометрии у пациентов с хронической сердечной недостаточностью на фоне приема препарата убидекаренона (коэнзима Q).

Материалы и методы. В исследование включены 53 пациента с хронической сердечной недостаточностью после перенесенного инфаркта миокарда. Пациенты были распределены в две группы: первая группа получала только оптимально подобранную стандартную терапию, вторая группа – дополнительно к оптимально подобранной медикаментозной терапии получала препарат убидекаренона («Кудевита»). Оценка митохондриального мембранного потенциала проводилась методом проточной цитометрии с применением йодистого пропидия и йодид 3,3'-дигексилоксакарбоцианина (DiOC6(3)). Определение содержания коэнзима Q в крови проводилось методом высокоэффективной жидкостной хроматографии с ультрафиолетовой детекцией.

Результаты. Выявлена прямая корреляционная зависимость между содержанием коэнзима Q в плазме крови и процентом DiOC-позитивных клеток (R = 0,39; p < 0,05) у пациентов с хронической сердечной недостаточностью. В группе пациентов, получавших только оптимально подобранную стандартную терапию, не выявлено статистически значимых различий в содержании коэнзима Q и процентном содержании DiOC-позитивных и DiOC-негативных клеток до начала и после терапии. В группе пациентов, получавших дополнительно препарат убидекаренона, после терапии наблюдалось статистически значимое увеличение доли DiOC-позитивных клеток и уменьшение доли DiOC-негативных клеток.

Заключение. Установлено повышение функциональной активности митохондрий у пациентов с хронической сердечной недостаточностью на фоне терапии препаратом убидекаренона. Метод проточной цитометрии может быть использован для оценки функционального состояния митохондрий и контроля эффективности применяемой терапии.

Ключевые слова: митохондрии, хроническая сердечная недостаточность, коэнзим Q, проточная цитометрия, митохондриальный мембранный потенциал

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Источник финансирования. Авторы заявляют об отсутствии финансирования при проведении исследования.

Соответствие принципам этики. Все участники исследования подписали информирование согласие. Исследование одобрено локальным этическим комитетом СЗГМУ им. И.И. Мечникова (протокол № 12 от 10.12.2014).

Для цитирования: Лобанова О.А., Гайковая Л.Б., Дадали В.А., Ермаков А.И., Кухарчик Г.А. Оценка функционального состояния митохондрий мононуклеарных лейкоцитов методом проточной цитометрии у пациентов с хронической сердечной недостаточностью под влиянием убидекаренона. *Бюллетень сибирской медицины.* 2022;21(2):90–96. https://doi.org/10.20538/1682-0363-2022-2-90-96.

INTRODUCTION

The use of ultrastructural analysis to study pathological processes in the myocardium has shown that mitochondria are the first to respond to any impact [1]. Identifying the nature and degree of mitochondrial damage in patients with chronic heart failure (CHF) is an important clinical and laboratory task, as it can determine the severity of the disease course [2, 3]. In addition, mitochondria are seen as a potential target for heart failure therapy [4–6].

To assess the functional state of mitochondria (M), there is a fairly large set of methods based on measuring the rate of oxygen uptake, autofluorescence of NADH and flavoproteins, the activity of M enzymes, and ATP levels [7]. However, not all methods can be used in clinical diagnostic laboratories. First of all, this is due to high complexity of research algorithms and analysis methods. One of the promising methods for studying the functional state of M is the assessment of changes in the mitochondrial membrane potential (MMP) using flow cytometry. The advantages of the method include a small amount of biological material required for the study and higher throughput capacity.

A drop in MMP can serve as an integral indicator of the functional state of M, since one of the most important functions of M is energy supply of cells, in which the respiratory chain plays an important role. The activity of the respiratory chain is accompanied by release of protons into the intermembrane space, which leads to the formation of a proton gradient, which triggers the activity of ATP synthase. Metabolic disorders, as well as the structure and integrity of the mitochondrial membrane, can ultimately result in a decrease in the MMP [8, 9].

Determination of MMP changes using flow cytometry is based on the use of special fluorescent dyes. Examples of such dyes are 3,3'-dihexyloxacarbocyanine iodide (DiOC6(3)) and propidium iodide (PI) [10, 11]. DiOC6(3) belongs to the group of lipophilic cationic dyes, which are called "mitochondrial probes" in the literature. Due to its lipophilic properties, DiOC6(3) is able to freely penetrate into the bilipid membranes of the cell, and, due to its cationic properties, this dye accumulates in areas with a high concentration of protons, that is, in mitochondria. This effect is accompanied by a change in the intensity of cellular fluorescence in the green region of the spectrum, which is recorded by flow cytometry [12]. If the proton concentration in the M is reduced, the dye will accumulate in them less efficiently, and, as a consequence, the intensity of its fluorescence will decrease. Thus, it is possible to distinguish between cells with efficiently functioning M and, consequently, high fluorescence intensity (DiOC6(3)-positive cells) and cells in which the functioning of M is impaired (DiOC6(3)-negative cells). Such cells have reduced fluorescence intensity.

In later stages of cell destruction, the integrity of the cell membrane is disrupted and, as a result, cell death occurs. To detect these late stages, another fluorescent dye, PI, is used, which cannot penetrate through cell membranes, but as it degrades, it begins to penetrate into the cell, accumulating in the cytoplasm and nucleus and interacting with DNA and RNA. As a result, the cell acquires the ability to fluoresce in the red region of the spectrum.

Thus, the method of flow cytometry using two fluorescent dyes makes it possible to detect not only cells with preserved functional state M, but also to identify cells at different stages of apoptosis, which is a consequence of developing mitochondrial dysfunction [13].

Given the complexity of obtaining human cardiac tissue for research purposes, one of the approaches for studying pathogenetic changes in the myocardium is to determine biochemical parameters in peripheral blood cells. The literature contains evidence of a correlation between changes in internal organs, including the myocardium, and changes in peripheral blood cells [14–16]. Thus, the works by E. Cortez et al. showed a correlation between changes in the biochemical parameters in peripheral blood mononuclear leukocytes and cardiomyocytes. The following parameters were determined: cellular respiration rate, carnitine palmitoyltransferase I, UCP 2, GLUT 1 [14].

The aim of the study was to assess the functional state of M of peripheral blood mononuclear leukocytes using flow cytometry in patients with CHF against the background of ubidecarenone (coenzyme Q) administration.

The objectives of the study were to determine the possibility of evaluating the effectiveness of ubidecarenone therapy by monitoring changes in the MMP in blood cells in CHF patients.

MATERIALS AND METHODS

The study included 53 patients with CHF who experienced myocardial infarction (MI) in the last 6 months before inclusion in the study. The patients were treated at St. Petersburg State Government-Funded Healthcare Institution "Elizavetinskaya Hospital". The study was conducted in accordance with the Declaration of Helsinki "Recommendations Guiding Physicians in Biomedical Research Involving Human

Subjects" and the requirements outlined in the main regulatory documents on clinical trials in the Russian Federation. The average age of the patients was 68 ± 8.1 years, including 28 men and 25 women. The diagnosis of CHF was based on the criteria of the Society of Heart Failure Specialists.

All patients were initially evaluated for the functional state of M of peripheral blood mononuclear leukocytes and the content of total coenzyme Q (CoQ) in the blood plasma. Besides, standard clinical and biochemical examinations were performed. The patients were then divided into two groups by block randomization (2 × 2). Group 1 included 28 patients who received optimally chosen standard therapy in accordance with the clinical guidelines for the diagnosis and treatment of coronary artery disease (CAD) and CHF. Group 2 encompassed 25 patients who received optimally chosen standard therapy and ubidecarenone ("Kudevite") at a dose of 120 mg per day (2 capsules (30 mg per capsule) in the morning and 2 capsules in the evening).

Patients of groups 1 and 2 were comparable in gender and age: the average age in group 1 was 70.0 ± 6.9 (56.0; 78.0) years, in group 2 – 66.8 ± 9.5 (49.0; 78.0) years ($p > 0.05$). In group 1, men accounted for 48%, in group 2 – for 52%. All studies were carried out twice: at hospitalization and 3 months after the start of the therapy.

To assess changes in MMP by flow cytometry, blood was collected in tubes containing EDTA. The leukocyte suspension was isolated using urografin. The obtained supernatant was used for the study. Then, a 20-fold DiOC6(3) solution (Invitrogen, USA) was added to 100 μl of the cell suspension, resulting in a final concentration of DiOC6(3) equal to 20 nM. The samples were then thoroughly mixed and incubated for 20 minutes at 37 °C in a 5% CO2 atmosphere in a dark place. The resulting cell suspension was added to 10 μl of a propidium iodide (PI) solution (Sigma-Aldrich, USA), obtaining a final concentration of PI equal to 1 μg / ml. The samples were then incubated for 10 minutes at room temperature in the dark place. Upon completion of the incubation, 200 μl of phosphate-buffered saline was added to the samples, and flow cytometric counting was performed. The obtained results were analyzed using the Kaluza ™ software (Beckman Coulter, USA).

The total plasma CoQ was determined by high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection on the Agilent 1200 series gradient system [17, 18]. CoQ determined in the blood plasma of group 1 patients before the start of the treatment was endogenous CoQ. In the patients of group 2, the determined CoQ was the sum of the endogenous CoQ and that obtained together with the drug.

As a drug containing ubidecarenone (CoQ), the drug "Kudevite" (PIK-PHARMA, Moscow) was used in the study. A feature of this pharmaceutical preparation is a high-tech substance ALL-Q produced in Switzerland, which provides optimal bioavailability of ubidecarenone. This substance has increased hydrophilicity, which makes it possible to convert poorly absorbed hydrophobic ubidecarenone into a water-soluble form that is optimal for absorption.

The research materials were statistically processed using parametric and nonparametric tests. Accumulation, correction, and systematization of the baseline data and visualization of the results were carried out in Microsoft Office Excel 2016 spreadsheets. Statistical analysis was carried out using the STATISTICA 10 software (StatSoft.Inc., USA).

Aggregates of quantitative variables with non-normal distribution were presented as the median and the interquartile range Me (Q_1–Q_3). To compare aggregates of independent variables with non-normal distribution, the Mann – Whitney U-test was used. In order to study the relationship between the quantitative data, the Spearman's rank correlation coefficient was used. The values of the correlation coefficient ρ were interpreted in accordance with the Chaddock scale (Table 1).

Table 1

Chaddock scale	
The values of the correlation coefficient r_{xy}	Characteristics of the strength of the correlation
less than 0.1	no correlation
0.1–0.3	weak
0.3–0.5	moderate
0.5–0.7	noticeable
0.7–0.9	high
0.9–0.99	very high

The differences were considered statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

The correlation analysis revealed a significant moderate positive correlation between the content of endogenous CoQ in the blood plasma (0.55 ± 0.11 μg / ml) and the percentage of DiOC6(3)-positive cells ($R = 0.39$, $p < 0.05$) in patients with CHF before the treatment. Low concentration of CoQ in patients with CHF is one of the factors leading to changes in mitochondrial processes and activation of processes

leading to cell death, which results in a decrease in the percentage of DiOC6(3)-positive cells where the functioning of M is preserved. The higher the concentration of CoQ, the more stable the mitochondrial membrane. Thus, the addition of ubidecarenone to therapy can improve the functional state of M.

In group 1 (patients who received only optimally chosen standard therapy), no statistically significant differences in the percentage of cells before and after the therapy were revealed (Table 2). In group 2 (patients who additionally received ubidecarenone), a statistically significant increase in the percentage of DiOC6(3)-positive cells and a decrease in the percentage of DiOC6(3)-negative cells were observed after the therapy.

Table 2

The content of DiOC6(3)-positive and DiOC6(3)-negative cells in patients with CHF before and after the treatment, %, *Me* (Q_1–Q_3)

Parameter	DiOC6(3)-positive cells	DiOC6(3)-negative cells, %
Group 0 (before the start of the treatment)	75.0 (67.0; 80.4)	21.5 (19.5; 32.9)
Group 1 (standard therapy)	77.0 (71.0; 85.4)	21.8 (14.4; 28.9)
Group 2 (standard therapy + “Kudevite”)	94.0 (80.0; 95.0)*	4.2 (4.0; 19.5)**

* $p = 0.025$ (when compared with group 0), $p = 0.044$ (when compared with group 1).
** $p = 0.031$ (when compared with group 0), $p = 0.043$ (when compared with group 1).

The correlation analysis in group 2 showed a significant negative correlation between the content of CoQ in the blood plasma and the baseline percentage of DiOC6(3)-negative cells ($R = – 0.45$, $p < 0.05$).

DiOC6(3)-positive cells exhibit high fluorescence in the green region of the spectrum, which is associated with active accumulation of the DiOC6(3) dye. This indicates preservation of MMP and, consequently, the main processes aimed at its formation. Thus, against the background of ubidecarenone therapy, an increase in the functional activity of M is observed. It is known that ubidecarenone, penetrating into cells, is involved in the work of the respiratory chain and participates in cellular energy supply [19, 20]. In addition, ubidecarenone has antioxidant properties and reduces production of reactive oxygen species (ROS). ROS, through the activation of MAP kinases, including p38 and p53, activate proapoptotic factors (Bax, Bak, and others) and contribute to opening of mitochondrial pores. A drop in ROS production leads to a decrease in the release of proapoptotic proteins from the mitochondrial matrix into the cytosol, suppression of apoptosis, and, as a consequence, a decrease in subsequent cell death. Thus, the functional activity of M, including the work of the respiratory chain, is preserved. As a result, the release of protons into the intermembrane space is restored, leading to restoration of the MMP.

Against the background of ubidecarenone therapy, a decrease in the percentage of DiOC6(3)-negative cells was observed, which may be associated with a decrease in ROS production and suppression of apoptosis intensity. Due to the increased concentration of protons in the intermembrane space of M, DiOC6(3) is more intensively accumulated in M, leading to an increase in the fluorescence intensity in the green region of the spectrum. At the same time, due to the preservation of the cell membrane structure following a decrease in the activity of lipid peroxidation, penetration of another dye (PI) into cells and its accumulation in them decreases. This is manifested through a decrease in the fluorescence intensity in the red region of the spectrum, which indicates a decrease in the number of cells at the stage of early apoptosis and dying cells.

CONCLUSION

Thus, flow cytometry revealed an increase in the functional activity of M in patients with CHF during ubidecarenone therapy, which is confirmed by a significant increase in the percentage of DiOC6(3)-positive cells. Determination of the functional state of M of peripheral blood mononuclear leukocytes using flow cytometry can be used to assess the functional state of M and monitor the effectiveness of therapy in patients with CHF.

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Acknowledgements

The authors would like to thank Ksenia A. Zagorodnikova, Cand. Sci. (Med.), Head of the Clinical Pharmacology Department, Clinical Pharmacologist, for assistance in conducting the chromatographic study.

Authors contribution

Lobanova O.A., Ermakov A.I. – carrying out of the research, analysis and interpretation of the data. Gaykovaya L.B. – conception and design. Dadali V.A. – final approval of the manuscript for publication. Kukharchik G.A. – selection of patients.

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Received 26.02.2020;
approved after peer review 25.03.2021;
accepted 25.05.2021