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Study of danazol active agent effect on Mcf10a breast cells redox phosphorylation

Relevance: Fibrocystic mastopathy is one of the most complex breast pathologies, occurring in about 60% of women. The causes and molecular mechanisms of cyst formation in fibrocystic mastopathy are not fully understood, and effective treatment methods are not widely implemented. However, there is evidence of the fibrocystic mastopathy malignization risk. We studied the molecular effect of the active agent danazol on the respiratory complex in mitochondria, which occur in various concentrations and are considered the cell energy center.

A method of permeation with special agents was developed to improve the cell membrane permeability in the intracellular proteins and nucleic acids analysis. The scientists aimed to study the Mcf10a cell line mitochondrial respiratory complex using the permeabilization method.

Purpose: To study the effect of different danazol concentrations on Mcf10a breast cells redox phosphorylation in women with fibrocystic mastopathy.

Results: Monitoring the redox phosphorylation process using complex respiratory substrates affecting the Mcf10A breast cells respiratory chain showed the danazol 30 μ M effect on the respiratory complex I.

Conclusions: According to oxygraphic records, 10 minutes' exposure to danazol 30 μ M after permeabilization with digitonin increased the Mcf10A cells' oxidative phosphorylation compared with the control. The respiratory rate decreased by 45% ten minutes after treatment with digitonin.

Keywords: fibrocystic mastopathy (FM), danazol, permeabilization.

Introduction: An increasing number of fibrocystic mastopathy cases has caused different studies and many clinical trials, which continue to this day. Various types of synthetic and natural medicines were developed based on those studies. However, till now, none of them has produced a lasting positive therapeutic effect [1]. The statistics evidence a growing frequency of fibrocystic mastopathy in women above 30 years.

Fibrocystic mastopathy is a benign change in the mammary gland in women, characterized by nodular formations. In order to timely detect and prevent complications of the disease, women are examined by a mammologist at local healthcare facilities twice a year [2, 3].

If a breast cyst increases in size and cancer is suspected, an aspiration biopsy is recommended to reveal the stage of cancer.

Certain drugs are usually administered to relieve pain. In fibrocystic mastopathy, depending on the size of the cyst, fluid is sucked off with special needles, and hormonal drugs are administered to reduce pain. Danazol is one of such active agents [4]. Regular use of this active agent decreases mammary gland hyperplasia in mastodynia. Androgens are used as es-

trogen antagonists in the treatment of mastopathy. Laboratory experiments in animals studied the ability of danazol to reduce the synthesis of some basic enzymes and gonadotropic hormones in ovarian steroidogenesis [5, 6]. This active agent is used in treating patients; however, they often refuse to take it due to side effects.

The main requirements for the drug, such as absorption and excretion, dosage, shelf life, are known. However, despite its widespread use in clinical practice, there is no information about the mechanisms of its action at the cellular level. Therefore, we have studied the effect of Mcf10A breast cells on energy metabolism, taking into account the effect of this active agent.

The purpose was to study the effect of different danazol concentrations on Mcf10a breast cells' redox phosphorylation in women with fibrocystic mastopathy.

Materials and methods: The study material included the danazol active agent in the concentrations of 0.25, 1, 3, 10, and 30 μ M, and Mcf10A breast cells taken from women with fibrocystic mastopathy [7].

Preparation of active agent concentrations. A special solution was prepared to ensure the pharmaceutical

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relevance of the active agent. The solution contained: PEG 400 (polyethylene glycol), 2-Pyrrolidone (a lactam compound soluble in water and alcohol) (15%), glycerin (5%), and propylene glycol (5%), ethanol (50%), and water (10%). Thus, special concentrations of the danazol active agent (USA, Pennsylvania) of 0.25, 1, 3, 10, and 30 μM were prepared for the experiment. Such concentrations were chosen because the impurities in the concentrations above 30 μM were prone to crystallization. In our study, mixtures of concentrations of the active agent were used following a specially approved protocol.

Msf10A cells were grown in 50-60 passages. Nutrient medium for cell growth contained: culture medium F12 DMEM/Hema (Cellgro, Mediatech, VA, USA), 5% FBS, EGF (epidermal growth factor) (100 $\mu\text{g/ml}$), hydrocortisone (1 mg/ml), insulin (10 mg/ml), and cholera toxin (1 mg/ml). We used Triton X-100, RNase, DMSO. Characteristics of F12 DMEM/Ham:

- DMEM (Dulbecco's Modified Eagle Medium)-modified breeding medium;
- product certification organization – Biowest;
- productID: L0090-500;
- sterility level – sterile;
- storage temperature – + 2/+ 8°C;
- shelf life – 24 months;
- amount of L-glutamine powder in the mixture – 0.365 g/l;
- color – pink solution.

The volume of chemical reagents used was according to the guidelines; during the study, each container with a solution was labeled with the composition and the time of production and stored in a freezer or refrigerator at a certain temperature.

The Msf10A mammary epithelial cells obtained from the American Culture Collection (ATCC, Manassas, USA) were taken from women with fibrocystic mastopathy (Drexel University, Laboratory of Mitochondrial Pathophysiology, Pennsylvania, USA). In the laboratory, those cells were stored following strict sanitary and hygienic guidelines. Msf10A cells were grown in 50-60 passages in the culture medium, containing 5% FBS (fetal bovine serum), L-glutamine F12 DMEM/Ham powder (Cellgro, Mediatech, VA, USA) culture medium with epidermal growth factor (EGF epidermal growth factor) (100 $\mu\text{g/ml}$), hydrocortisone (1 mg/ml), insulin (10 mg/ml), and cholera toxin (1 mg/ml).

Results: There are two types of reagents for permeabilization: (1) organic solvents (methanol, etha-

nol, acetone) dissolve membranes and fats and promote protein coagulation and permeabilization; (2) detergents such as saponin, Triton-X-100, Tween-20, and others make holes in the cell membrane. *In the study, we studied the respiration rate of Msf10A mammary gland cells using the OROBOROS Oxygraph-2K device (Innsbruck, Austria) at 37°C with a certain concentration danazol active agent.* Danazol concentrations of 0.25, 1, 3, 10, and 30 μM were used after dissolution, sedimentation, and collection of experimental cultured cells by trypsinization and centrifugation. The study failed to show clear results on danazol concentrations of 0.25, 1, 3, 10, and 30 μM ; therefore, the maximum efficient concentration of danazol active agent in mammary gland Msf10A cells was 30 μM (as a control concentration).

Respiration rate was measured for million cells per second. Permeabilization techniques were used to monitor the chain of individual respiratory enzyme activity (Figure 1).

The permeabilization methods used in the study followed the protocol guidelines. 10 μM glutamate, 10 μM pyruvate and 2 μM malate, 1 mg/ml rotenone, 10 μM succinate, 2.5 μM antimycin and 1 mM ascorbate/0.3 mM TMPD (tetramethyl phenylenediamine) were used to determine the activity of respiratory enzymes.

One of the main stages in the cell line study was to determine the cellular respiration rate. Digitonin used to control the activity of respiratory enzymes is an organic glucoside. Its aglycones are derivatives of spirostane aglycones. In our study, we used digitonin as a detergent. Differences between digitonin, succinate, glutamate, malate, and rotenone-stimulated respiration were evident in respirometric analyzes showing mitochondrial size, respiratory enzymes, and low oxidative activity.

In the Complex II study, titration with succinate was performed to assess the maximum respiratory activity.

No significant differences in cellular respiration were observed in the presence of glutamate (glutamic acid), pyruvate, and malate (malic acid) after altering the cell membrane permeability with digitonin. However, the mitochondrial membrane remained damaged inside the cells perforated with digitonin. The cells continued breathing at a high rate for a long time in the presence of exogenous succinate. However, soon, the respiratory rate decreased due to the disturbance

of the intracellular environment. Figure 1 shows that the Complex I inhibitor was treated with rotenone (1 mg/ml) and succinate. Oxygraphic records in Figure 1A show an increase in danazol concentration by 120% within 10 minutes after adding a 30 μ M concentration

after permeabilization. This evidences the effect 30 μ M danazol concentration has on the Msf10A cells' oxidative-phosphorylation mechanism. After 10 minutes after digitonin was added, the respiratory rate dropped to 45% (* $p \leq 0.05$).

Here, the X-axis shows the oxygen concentration in μ Mol/ml, and the Y-axis shows the time difference. Abbreviated data on the substrates of the respiratory complex (Com I, NADH dehydrogenase) – 10 mM glutamate, 10 mM pyruvate, and 2 mM malate (G/P/M), 1 mg/ml Rotenone (Rot) as an inhibitor of Complex I, the substrate of Complex II (Com II, succinate dehydrogenase) – 10 mM succinate (Suc), 2.5 mM Antimycin A (Ant) as an inhibitor of Complex II, and the substrate of Complex IV (Com IV, cytochrome c oxidase) – 1 mM Ascorbate with 0.3 mM TMPD (Asc/TMPD). Bold lines indicate the difference in the respiratory activity of Complex 1.

Figure 1 – The effect of a 30 μ M danazol concentration on Msf10A cells oxidation-phosphorylation mechanism. A: Oxygraphic records after permeabilization with digitonin. B: Oxygraphic records after adding a 30 μ M danazol concentration.

Conclusion: The study object was the processes in cell mitochondria. This organoid plays an important role in in-cell energy metabolism. It participates in cellular respiration and releases energy that other structures can use. That is why mitochondria are also called “an energy environment of the cell, a power plant.” Mitochondria oxidative phosphorylation produces a large amount of active endogenous cellular oxygen. Literature data demonstrate that a certain part of Complex I strongly regulates the production of reactive oxygen forms under normal physiological conditions. Besides, respiratory enzymes form electron transport chains or respiratory chains and contain about 40 different proteins arranged in a certain order depending on the degree of change in the redox potential, i.e., the ability to add (decrease) or transfer (oxidize) electrons. Therefore, these studies show that Complex I is the direct target for danazol active agent in the cell structure since it decreases with an increase in oxygen consumption in the respiratory chains of mitochondria I, III, and IV.

Indications for clinical use of danazol inform that it suppresses pathological processes. However, wom-

en refuse to take this active agent for breast pathology (mastopathy, fibrocystic mastopathy) due to many side effects. Therefore, for a controlled assessment of its mechanism of action at the cellular level, the scientists focused their experiments on the processes in mitochondria as one of the main cell organelles. In the latest presented research results, the focus was on the oxidative-phosphorylating function of the cell, the cell cycle, and other processes such as the production of hydrogen peroxide.

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ТҰЖЫРЫМ

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Mcf10A сүт безі клеткаларының тотығу-фосфорлануына даназол препаратының әсер ету механизмін зерттеу

Өзектілігі: Фиброзды-кистозды мастопатия (ФКМ) әйелдердің 60%-ға жуығында кездесетін сүт безінің күрделі патологияларының бірі болып табылады. Фиброзды-кистозды мастопатияның киста түзілу себептері мен молекулалық механизмдері толыққанды зерттелінбеген және тиімді емдеу жолдары кеңінен енгізілмеген. Сонымен бірге фиброзды-кистозды мастопатияның қатерлі ісікке айналу қаупі бар деректер де келтіріледі. ФКМ кезінде қолданылатын белсенді заттың молекулалық деңгейдегі әсер ету механизмін зерттеу барысында, осы белсенді заттың әртүрлі концентрацияларын алып, жасушаның энергетикалық орталығы болып есептелетін митохондриядағы тыныс алу кешеніне әсері зерттелді.

Жасуша мембранасының өткізгіштігін жақсарту үшін жасуша ішілік ақуыздар мен нуклеин қышқылдарын талдау кезінде арнайы заттармен пермебилизация әдісі жасалды. Зерттеушілер пермебилизация әдісін қолдана отырып, Mcf10A жасушалық желісінің митохондриялық тыныс алу кешенін зерттеу міндетін қойды.

Зерттеудің мақсаты: Фиброзды-кистозды мастопатиясы бар әйелдерден алынған Mcf10A сүт безі жасушаларының тотығу-фосфорлануына даназол препаратының әсер ету механизмін белсенді затының әртүрлі концентрациясының әсерін зерттеу.

Нәтижелер: Mcf10A сүт безі жасушаларының тыныс алу тізбегіне әсер ететін тыныс алу кешенінің субстраттарын қолдана отырып, тотығу фосфорлану процесін бақылау кезінде даназол 30мкМ белсенді заттың концентрациясы I тыныс алу кешенінің кешеніне әсер ететіні анықталды.

Қорытындылар: 30мкМ концентрациясындағы даназолдың әсер етуші затының Mcf10A жасушаларына әсері кезінде біз дигитонинмен пермебилизациядан кейін оксиграфиялық жазбаларда көрсетілгендей бақылаумен салыстырғанда 10 минут ішінде тотығу фосфорлануының күшеюін байқадық. Дигитонин қосылғаннан кейін 10 минуттан кейін тыныс алу жиілігі 45% төмендеді.

Түйінді сөздер: Фиброзды-кистозды мастопатиясы, белсенді ингредиент даназол, пермебилизация.

АННОТАЦИЯ

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Исследование влияния действующего вещества даназол на механизм окислительно-восстановительного фосфорилирования клеток молочной железы Mcf10A

Актуальность: Фиброзно-кистозная мастопатия (ФКМ) – одна из наиболее сложных патологий молочной железы – встречается у примерно 60% женщин. Причины и молекулярные механизмы образования кисты при ФКМ полностью не изучены, отсутствуют широко применимые эффективные методы лечения. Вместе с тем приводятся данные о риске превращения ФКМ в злокачественную. В ходе изучения механизма действия действующего вещества даназол, применяемого при ФКМ, было изучено влияние этого препарата на молекулярном уровне на дыхательный комплекс в митохондриях, которые встречаются в различных концентрациях и считаются энергетическим центром клетки.

Для улучшения проницаемости клеточной мембраны при анализе внутриклеточных белков и нуклеиновых кислот разработан метод пермебилизации специальными веществами. Исследователи поставили перед собой задачу изучить митохондриальный дыхательный комплекс клеточной линии Mcf10A, используя метод пермебилизации.

Цель исследования: Изучить влияние различных концентраций действующего вещества даназол на механизм окислительно-восстановительного фосфорилирования клеток молочной железы Mcf10A, полученных от женщин с фиброзно-кистозной мастопатией.

Результаты: При мониторинге процесса окислительного фосфорилирования с использованием субстратов респираторного комплекса, влияющих на дыхательную цепь клеток молочной железы Mcf10A, было обнаружено, что концентрация действующего вещества даназол 30мкМ влияет на комплекс дыхательного комплекса I.

Выводы: При влиянии действующего вещества даназол в концентрации 30мкМ на клетки Mcf10A мы наблюдали усиление окислительного фосфорилирования в течение 10 минут после добавления в сравнении с контролем, как показано в оксиграфических записях после пермебилизации дигитонином. Через 10 минут после добавления дигитонина частота дыхания снизилась на 45%.

Ключевые слова: Фиброзно-кистозная мастопатия (ФКМ), действующее вещество даназол, пермебилизация.