

Biological effects of mitochondrial therapy: preventing development of myocardial infarction and blocking metastatic aggression of B16/F10 melanoma

Oleg I. Kit¹, Elena M. Frantsiyants¹, Alla I. Shikhlyarova¹, Irina V. Neskubina^{1*}, Irina V. Kaplieva¹, Natalia D. Cheryarina¹, Alexandra A. Vereskunova², Lidia K. Trepitaki¹, Yulia A. Pogorelova¹, Valeria A. Bandovkina¹, Ekaterina I. Surikova¹, Polina S. Kachesova¹, Elena A. Sheiko¹, Inga M. Kotieva^{1,2}, Marina A. Gusareva¹, Roza G. Luganskaya¹, Ekaterina S. Bosenko¹

¹National Medical Research Centre for Oncology, Rostov-on-Don, Russia, 344037, Rostov-on-Don, 14 liniya, 63, building 8; e-mail: super.gormon@yandex.ru

²Rostov State Medical University, 29 Nakhichevsky lane, Rostov-on-Don 344022, Russian Federation

* Corresponding author:
super.gormon@yandex.ru

Abstract

The aim is to evaluate the physiological parameters of the efficacy of cardiac mitochondria transplantation in male mice with chronic neurogenic pain and B16/F10 melanoma growth.

Materials and methods. Male mice (n=37) of the C57BL/6 line were used in the research work. The animals covered experimental groups as follows: mice with chronic neurogenic pain (CNP) + B16/F10 melanoma (n=27); mice with CNB + B16/F10 melanoma + mitochondrial therapy (MC therapy) (n=10). Mitochondria were isolated from the heart of an intact rat with the use of differential centrifugation. An introduction of mitochondria to mice was carried out daily intraperitoneally at a dose of 3.3 mg of protein for 3 weeks.

Statistical analysis of the results is carried out with the Statistica 10.0 software.

Results. On day 21 (week 3) of the experiment, macroscopically in the melanoma tissue in the group of animals with MC therapy, there were 2.5 times more necrosis cases than in the group without MC therapy. During the examination of the internal organs, no metastases were detected in the animals treated with MC therapy, while in 100% of the animals without MC therapy metastases were found in the lungs and in 95% of them in the spleen. In the animals received MC therapy, there was no dam-

age to the heart muscle in 75% of the cases, while in the group of the animals without MC therapy, the presence of lesions in the form of bruises on the surface of the heart was macroscopically detected in 100% of the animals.

Conclusion. Thus, intraperitoneal transplantation of intact heart mitochondria contributed to the prevention of myocardial infarction and metastases to internal organs in the C57BL/6 male mice with B16/F10 melanoma growing against the background of chronic neurogenic pain.

Keywords

Mitochondrial therapy, Mitochondria, Heart, Myocardial infarction, B16/F10 melanoma, Chronic neurogenic pain, Male mice

Imprint

Oleg I. Kit, Elena M. Frantsiyants, Alla I. Shikhlyarova, Irina V. Neskubina, Irina V. Kaplieva, Natalia D. Cheryarina, Alexandra A. Vereskunova, Lidia K. Trepitaki, Julia A. Pogorelova, Valeria A. Bandovkina, Ekaterina I. Surikova, Polina S. Kachesova, Elena A. Sheiko, Inga M. Kotieva, Marina A. Gusareva, Roza G. Luganskaya, Ekaterina S. Bosenko. Biological effects of mitochondrial therapy: preventing development of myocardial infarction and blocking metastatic aggression of B16/F10 melanoma. *Cardiometry*; Issue 22; May 2022; p. 50-55; DOI: 10.18137/cardiometry.2022.22.5055; Available from: <http://www.cardiometry.net/issues/no22-may-2022/biological-effects-mitochondrial-therapy>

Introduction

Cardiovascular diseases (CVD) are considered one of the main causes of death in population [1]. Numerous complex factors are involved in the onset and progression of heart diseases; however, in recent years, mitochondrial dysfunction has been recognized as a hallmark in cardiac physiopathology [2, 3].

The heart is an organ with high energy requirements, therefore, it is not surprising that mitochondria occupy 30% of the total volume of cardiomyocytes and generate approximately 95% of ATP in the body [4]. Mitochondria are organized into three different subgroups based on their function and location: subarcolemmal (under the sarcolemma), perinuclear (around the nucleus), and interfibrillar (between myofibrils) mitochondria. The interfibrillar mitochondria are the most abundant type of them, and they are involved in the production of ATP to support myocyte contraction by regulating Ca²⁺ signaling during the cardiac excitation-contraction coupling [4, 5].

Mitochondria transplantation is an innovative strategy to treat mitochondrial dysfunction in order to overcome the limitations imposed by agent-based therapy methods. Mitochondrial transplantation is targeted at the transfer of the functional exogenous mitochondria into mitochondrially defective cells to repair or prevent mitochondrial diseases, or more simply, to replace the old “engine” with a new one in order to restore the performance thereof. Artificial mitochondrial transfer was first attempted by Clarke and Shay. They co-incubated mitochondria purified from cells resistant to chloramphenicol and tetracycline, and from mammalian cells sensitive to this sort of antibiotics. As a result, mitochondria-mediated transfer of antibiotic resistance through endocytosis was confirmed [6]. This phenomenon of organelle delivery to recipient cells can be applied to mitochondrial diseases. Replacement of non-functional mitochondria in damaged tissues or cells with functional ones may represent a new approach to the treatment of many diseases.

Several *in vitro* studies have shown that the intercellular transfer of mitochondria occurs in a natural manner. When DsRed-labeled mitochondria isolated from mesenchymal cells (EMCs) derived from human uterine endometrial glands were co-incubated with isogenic EMCs for 24 h, an accumulation of exogenous mitochondria in the cytoplasm of recipients was observed using live fluorescent cell imaging [7]. In another study, it is also noted that the xenogenic transfer of mitochondria isolated from the mouse liver tissue into some human cells deprived of functional mitochondria (ρ^0 cells) restores respiratory function [8]. These results prove the possibility of treating mitochondrial diseases with artificial mitochondrial transplantation.

Previously, it was shown in experimental animals that in the presence of a malignant process in the body, a mitochondrial dysfunction occurs, which affects the entire organism [9, 10]. Most often, the tumor process develops against the background of some comorbid pathology [11]. When studying melanoma against the background of chronic pain in experimental animals, an aggravation of the malignant process was observed that was expressed by heavier metastasizing, often to atypical sites, by shortening the life spans of animals and a larger volume of the tumor foci [12]. It has been found that just in case of the combination of two pathological processes (chronic neurogenic pain and B16/F10 melanoma), starting from the first week of the tumor growth, infarction events occur in animals,

increasing in their the number with the tumor growth [13,14]. Based on the above facts and reference literature data, it seems necessary to use transplantation of intact heart mitochondria with an assessment of the possible control of myocardial infarction in animals.

The aim hereof is to evaluate the physiological parameters of the mitochondrial transplantation efficacy aimed at preventing myocardial infarction in male mice with chronic neurogenic pain and the B16/F10 melanoma growth.

Materials and methods

Male mice ($n=47$) of the C57BL/6 line, aged 8 weeks, with an initial individual body weight of 28–30 g, were used in our research work. The animals were delivered by the Andreevka Research Center for Biomedical Technologies at the Federal Medical and Biological Agency (Moscow Region). The experimental groups were composed as follows: the mice with chronic neurogenic pain (CNP) + B16/F10 melanoma ($n=27$); the mice with CNP + B16/F10 melanoma + mitochondrial therapy (MC therapy) ($n=20$).

The laboratory animals (the mice) were kept under natural light conditions with free access to water and food. Work with animals was carried out in accordance with the rules of the “European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes” (Directive 86/609/EEC) as well as in accordance with the International Guiding Principles for Biomedical Research Involving Animals and Order No. 267 “Approval of the Rules of Laboratory Practice” dated June, 19, 2003 issued by the Ministry of Health of the Russian Federation.

Model of chronic neuropathic pain

All manipulations with animals were carried out in a box. Tools, utensils and hands were disinfected in the conventional way, applying all asepsis requirements.

The model of chronic neurogenic pain (CNP) was reproduced by applying a ligature to the sciatic nerve from both sides under xylazoletyl anesthesia [15]. Anesthesia: xyl-zoletyl, 10 minutes before the main anesthesia; premedication: xylazine (Xyla preparation) intramuscularly, at a dose of 0.05 ml/kg of body weight (according to the instructions), then after 10 minutes Zoletil-50 was administered at a dose of 10 mg per 100 g of body weight. On post-operative day 14, mechanical allodynia and hyperalgesia were measured.

2 weeks after the reproduction of chronic neurogenic pain, B16/F10 melanoma was transplanted into the C57BL/6 line mice by standard subcutaneous injection of tumor suspension under the right shoulder blade in a volume of 0.5 ml of cell suspension in a 1:10 dilution with saline solution. The tumor strain of mouse melanoma B16/F10 was ordered from the Russian National Medical Research Center of Oncology named after N.N.Blokhin, at the Ministry of Health, the Russian Federation.

Isolation of mitochondria

An intact rat was sacrificed with a guillotine, and the heart was harvested on ice and perfused with an ice-cold sterile 0.9% KCl solution. Mitochondria were isolated using differential centrifugation with a high-speed refrigerated centrifuge Avanti J-E, BECMAN COULTER, USA according to the method by Egorova M.V. and Afanasiev S.A. 2011 [16]. To destroy the intercellular bonds, the cell wall and plasma membranes, mechanical processing of tissues was utilized with grinding with scissors and homogenization in a glass homogenizer with a Teflon pestle (the Potter-Elvehjem homogenizer). Per gram of tissue, 10 ml of sterile isolation medium (0.22 M mannitol, 0.3 M sucrose, 1 mM EDTA, 2 mM TRIS-HCL, 10 mM HEPES, pH 7.4) was added. The tissues were homogenized and centrifuged for the first time for 10 min at a speed of 1000 g, at a temperature 0-2°C, the second and third centrifugations were carried out at 20000 g, 20 min, at a temperature 0-2°C. Between the centrifugation procedures, the mitochondrial pellet was resuspended in the isolation medium. Mitochondria were further purified from lysosomes, peroxisomes, melanosomes, etc. by centrifugation in a 23% Percoll gradient. The suspension of the subcellular structures was layered on a Percoll gradient, centrifuged for 15 min at 21000 g, after which separation into 3 phases was observed; the lower layer of mitochondria was left and resuspended in the isolation medium. The next washing of mitochondria was carried out by centrifugation for 10 min at 15000 g, at a temperature 0-2°C. Mitochondrial samples were diluted with a 0.9% NaCl solution to a protein concentration of 3.3 mg of protein in 0.3 ml of the saline solution.

Conducting bio-therapy with mitochondria

24 hours after the B16/F10 melanoma inoculation, the mice were intraperitoneally injected with freshly

isolated heart mitochondria (3.3 mg of protein per animal in 0.3 ml of the saline solution). Further, mitochondrial therapy was carried out daily until the end of the experiment - 3 weeks of the B16/F10 melanoma growth.

The male C57BL/6 line mice with B16/F10 melanoma, growing against the background of chronic neurogenic pain, were used as the reference, which were injected intraperitoneally with 0.3 ml of the saline solution daily.

The animals were decapitated 3 weeks after the B16/F10 melanoma inoculation.

Statistical processing of the results was carried out using the Statistica 10.0 software. The data obtained were analyzed for the compliance of the distribution of signs with the normal distribution law using the Shapiro-Wilk test (for small samples). The comparison of quantitative data in groups (independent samples) was performed using a parametric Student's t-test. Table data are presented as $M \pm m$, where M is the arithmetic mean, m is the standard error of the mean; $p < 0.05$ was taken as the level of statistical significance. The results obtained were statistically processed in compliance with the general recommendations for medical research.

Results

The collected pathological parameters of the tumor growth dynamics under the conditions of the growth of B16/F10 melanoma against the background of chronic neurogenic pain during bio-therapy with heart mitochondria in the C57BL/6 male mice are presented in Table 1 herein.

The subcutaneous tumor in the males became detectable on day 7 after the inoculation of the B16/F10 melanoma cells. At the same time, the tumor volume in the group of the males, who received MC therapy during that period, was 1.4 times less ($p < 0.05$) than that recorded in the group of the animals without MC therapy. At that stage of the tumor growth, other pathological parameters characterizing the tumor process were identical in all studied groups of the animals. At the end of the experiment on day 21 (week 3), macroscopically, a greater number of necrosis cases in the melanoma tissue (2.5 times greater) was found in the group of animals with MC therapy than it was the case with the group without MC therapy. During the re-examination of all organs, the absence of metastases was recorded in the animals receiving MC therapy,

Table 1
Dynamics of tumor growth in C57BL/6 male mice against the background of mitochondrial therapy

| Groups | Chronic neurogenic pain + B16/F10 | | Chronic neurogenic pain + B16/F10+ Mitochondrial therapy | |
|-------------------------------------|-----------------------------------|---|--|----------------|
| | week 1 n=17 | week 3 n=10 | week 1 n=10 | week 3 n=10 |
| Mean tumor size, cm ³ | 0.06±0.01 | 3.00±0.58 | 0.042±0.007 ¹ | 2.65±0.97 |
| Minimum tumor size, cm ³ | 0.0001 | 0.45 | 0.0001 | 1.24 |
| Maximum tumor size, cm ³ | 0.125 | 6.5 | 0.12 | 5.47 |
| Heart damage | Not found | 100% | Not found | 25% |
| Metastases | Not found | Found (lungs in 100%, spleen in 95% of cases) | Not found | Not found |
| Necrosis | Not found | 20% | Not found | 50% |

Note: 1 – statistically significant differences relative to the group without mitochondrial therapy.

while in the animals without MC therapy there were metastases found (the lungs, the spleen 95%). Particular attention was drawn to the fact that in 75% of the cases there was no damage to the heart muscle in the animals who received MC therapy, while in the group of the animals without MC therapy, the presence of damage in the form of maroon spots on the surface of the heart was macroscopically detected in 100% of the rodents. It should be recognized that it was not possible to obtain a pronounced anti-tumor effect upon the intraperitoneal injection of heart mitochondria on the primary focus, however, the general anti-tumor effect consisted in the full absence of metastases to somatic organs. And most importantly, a convincing result was obtained in the prevention of myocardial infarction that was the aim of our research work.

Discussion

Currently, more and more experimental and clinical evidence data indicate a favorable therapeutic effect resulting from mitochondrial therapy, while methods of the transplantation, as well as types of transplantation (autologous, allogeneic, xenogenic) are extensively studied. Thus, the therapeutic effect of mitochondrial transplantation on a model of a heart disease was evaluated. McCully J.D. (2003) has demonstrated that ischemia causes mitochondrial dysfunction and suppresses cell viability and recovery of cardiomyocyte functions after reperfusion [17]. Other researchers isolated mitochondria from tissue not affected by ischemia and then injected them into the ischemic zone immediately before reperfusion. This resulted in a significant improvement in the post-ischemic functional recovery and the cell viability [18]. Continuing

their developments in mitochondrial therapy, the researchers compared the mitochondrial localization by two different delivery pathways: a direct injection of human cardiac fibroblast mitochondria into the ischemic tissue in the rabbit heart and the vascular delivery of mitochondria through the coronary arteries at the beginning of reperfusion. As expected, the directly injected mitochondria were localized close to the delivery site, while the vascularly delivered mitochondria were found widely scattered throughout the heart, with both delivery methods providing the cardiac protection against ischemia-reperfusion injury [19].

The therapeutic effects of mitochondrial transplantation have been confirmed in experimental animal models as well as clinical trials. A group of doctors performed autologous transplantation of mitochondria isolated from non-ischemic rectus abdominis muscles into the area of the damaged myocardium in patients with ischemia-reperfusion injury. As a result, four of five patients experienced recovery of the ventricular function, and no short-term complications such as arrhythmia, intramyocardial hematoma, or scarring associated with mitochondrial transplantation were observed [20].

The autologous mitochondrial transplantation has not been shown to elicit any immune response in various animal models. They also investigated the immune response associated with the allogeneic mitochondrial transplantation. The authors administered mitochondria isolated from the gastrocnemius and quadriceps femoris muscles to syngeneic or allogeneic mice by a single and sequential intraperitoneal injection. As a result, there was no direct or indirect, acute or chronic alloreactivity, immunological reactivity of certain classes

of T-lymphocytes against transplanted mitochondria, or reactions of damage-associated molecular pattern molecules (DAMPs) to single or sequential injections of autologous or allogeneic mitochondria [21].

In our experiment, the allogeneic intraperitoneal transplantation of heart mitochondria was used, and we believe that the spread of mitochondria with the intraperitoneal injection can lead to a “settlement” of the organelles at the internal organs, as well as to penetration thereof into the circulatory and lymphatic systems, since large circulatory and lymphatic vessels are concentrated in the abdominal area.

Conclusion

Thus, the intraperitoneal transplantation of heart mitochondria has contributed to the prevention of myocardial infarction in the C57BL/6 line male mice with the B16/F10 melanoma growing against the background of chronic neurogenic pain. It is necessary to note another important effect of the heart mitochondrial transplantation, namely, a complete blocking of metastatic lesions of the internal organs, while in the animals of the reference group, metastases in the lungs are observed in 100% of the cases and in the spleen in 95% of the cases. This experimental project to identify the biological effects of transplantation of functionally active mitochondria into animals with melanoma growing against the background of chronic neurogenic pain is a pilot project. Nevertheless, it can serve as the basis for some large programs to address the use of MC therapy in oncology and other treatment fields, with further in-depth study of the mechanisms of the normal mitochondria action on pathological processes in the organism.

Statement on ethical issues

Research involving people and/or animals is in full compliance with current national and international ethical standards.

Conflict of interest

None declared.

Author contributions

The authors read the ICMJE criteria for authorship and approved the final manuscript.

References

1. Virani SS, et al. Heart Disease and Stroke Statistics-2020 Update: A Report From the American Heart

Association. *Circulation*. 2020;141:e139–e596. doi: 10.1161/CIR.0000000000000757.

2. Brown DA, et al. Expert consensus document: Mitochondrial function as a therapeutic target in heart failure. *Nat. Rev. Cardiol*. 2017;14:238–250. doi: 10.1038/nrcardio.2016.203.

3. Bonora M, et al. Targeting mitochondria for cardiovascular disorders: Therapeutic potential and obstacles. *Nat. Rev. Cardiol*. 2019;16:33–55. doi: 10.1038/s41569-018-0074-0.

4. Cao JL, et al. Role of mitochondrial Ca²⁺ homeostasis in cardiac muscles. *Arch. Biochem. Biophys*. 2019; 663: 276–287. doi: 10.1016/j.abb.2019.01.027.

5. Kohlhaas M, Nickel AG, Maack C. Mitochondrial energetics and calcium coupling in the heart. *J. Physiol*. 2017; 595:3753–3763. doi: 10.1113/JP273609.

6. Clark MA, Shay JW. Mitochondrial transformation of mammalian cells. *Nat. Cell Biol*. 1982;295:605–607. doi: 10.1038/295605a0.

7. Kitani T, et al. Direct Human Mitochondrial Transfer: A Novel Concept Based on the Endosymbiotic Theory. *Transplant. Proc*. 2014; 46: 1233–1236. doi: 10.1016/j.transproceed.2013.11.133.

8. Katrangi E, et al. Xenogenic transfer of isolated murine mitochondria into human rho0 cells can improve respiratory function. *Rejuvenation Res*. 2007;10:561–570. doi: 10.1089/rej.2007.0575.

9. Kit OI, et al. Influence of B16/F10 melanoma development variant on BCL-2 content in mitochondria of cells of various organs of female mice. *Bulletin of Siberian Medicine*. 2021;20(3):46-53. [in Russian]

10. Kit OI, et al. The processes of self-organization of mitochondria during the growth of experimental tumors under conditions of chronic neurogenic pain. *News of higher educational institutions. North Caucasian region. Series: Natural Sciences*. 2019; 2 (202):97-105. [in Russian]

11. Leppert W, Zajackowska R, Wordliczek J, Dobrogowski J, Woron J, Krzakowski M, Pathophysiology and clinical characteristics of pain in most common locations in cancer patients. *J. Physiology and Pharmacology*. 2016. 67(6). P. 787-799.

12. Kit OI, et al. The effect of chronic neuropathic pain on the course of the malignant process of B16/F10 melanoma in male mice. *News of higher educational institutions. North Caucasian region. Series: Natural Sciences*. 2019; 1 (201):106-11. [in Russian]

13. Frantsiyants EM, et al. Content of apoptosis factors and self-organization processes in the mitochondria

- of heart cells in female mice C57BL/6 under growth of melanoma b16 / f10 linked with comorbid pathology. *Cardiometry*. 2021; 18:121-30.
14. Frantsiyants EM, et al. The functional state of mitochondria of cardiomyocytes in a malignant process against the background of comorbid pathology in the experiment. *South Russian journal of oncology*. 2021; 2(3):13-22. [in Russian]
15. Kotieva I.M., et al. An increase in the degree of malignancy of melanoma against the background of chronic pain in female mice. *Malignant tumors*. 2017;7(3S1):123-124. [in Russian]
16. Egorova M.V., Afanasiev S.A. Isolation of mitochondria from cells and tissues of animals and humans: Modern methodological techniques. *Siberian medical journal*. 2011; 26(1-1):22-28 [in Russian]
17. Levitsky S, et al. Mitochondrial DNA deletions in coronary artery bypass grafting patients. *Eur. J. Cardiothorac. Surg.* 2003; 24: 777–784. doi: 10.1016/S1010-7940(03)00501-3.
18. McCully JD, et al. Injection of isolated mitochondria during early reperfusion for cardioprotection. *Am. J. Physiol. Circ. Physiol.* 2009; 296: H94–H105. doi: 10.1152/ajpheart.00567.2008.
19. Cowan DB, et al. Intracoronary Delivery of Mitochondria to the Ischemic Heart for Cardioprotection. *PLoS ONE*. 2016; 11:e0160889. doi: 10.1371/journal.pone.0160889.
20. Emani SM, et al. Autologous mitochondrial transplantation for dysfunction after ischemia-reperfusion injury. *J. Thorac. Cardiovasc. Surg.* 2017; 154: 286–289. doi: 10.1016/j.jtcvs.2017.02.018.
21. Ramirez-Barbieri G, et al. Alloreactivity and allorecognition of syngeneic and allogeneic mitochondria. *Mitochondrion*. 2019; 46:103–115. doi: 10.1016/j.mito.2018.03.002.