ISSN 0006-2979, Biochemistry (Moscow), 2024, Vol. 89, No. 7, pp. 1336-1348 © Pleiades Publishing, Ltd., 2024.

Differences in the Effect of Beta-Hydroxybutyrate on the Mitochondrial Biogenesis, Oxidative Stress and Inflammation Markers in Tissues from Young and Old Rats

Veronika V. Nesterova[1], Polina I. Babenkova[1], Anna A. Brezgunova[2], Natalia A. Samoylova[1], Irina S. Sadovnikova[1], Dmitry S. Semenovich[2], Nadezda V. Andrianova[2], Artem P. Gureev[1,3], and Egor Y. Plotnikov[2,a]*

[1]*Voronezh State University, 394018 Voronezh, Russia*
[2]*Belozersky Institute of Physical-Chemical Biology, Lomonosov Moscow State University, 119991 Moscow, Russia*
[3]*Voronezh State University of Engineering Technology, 394036 Voronezh, Russia*
[a]*e-mail: plotnikov@belozersky.msu.ru*

Received May 9, 2024
Revised June 13, 2024
Accepted June 19, 2024

Abstract—One of the therapeutic approaches to age-related diseases is modulation of body cell metabolism through certain diets or their pharmacological mimetics. The ketogenic diet significantly affects cell energy metabolism and functioning of mitochondria, which has been actively studied in various age-related pathologies. Here, we investigated the effect of the ketogenic diet mimetic beta-hydroxybutyrate (BHB) on the expression of genes regulating mitochondrial biogenesis (*Ppargc1a*, *Nrf1*, *Tfam)*, quality control (*Sqstm1*), functioning of the antioxidant system (*Nfe2l2*, *Gpx1*, *Gpx3*, *Srxn1*, *Txnrd2*, *Slc6a9*, *Slc7a11*), and inflammatory response (*Il1b*, *Tnf*, *Ptgs2*, *Gfap*) in the brain, lungs, heart, liver, kidneys, and muscles of young and old rats. We also analyzed mitochondrial DNA (mtDNA) copy number, accumulation of mtDNA damage, and levels of oxidative stress based on the concentration of reduced glutathione and thiobarbituric acid-reactive substances (TBARS). In some organs, aging disrupted mitochondrial biogenesis and functioning of cell antioxidant system, which was accompanied by the increased oxidative stress and inflammation. Administration of BHB for 2 weeks had different effects on the organs of young and old rats. In particular, BHB upregulated expression of genes coding for proteins associated with the mitochondrial biogenesis and antioxidant system, especially in the liver and muscles of young (but not old) rats. At the same time, BHB contributed to the reduction of TBARS in the kidneys of old rats. Therefore, our study has shown that administration of ketone bodies significantly affected gene expression in organs, especially in young rats, by promoting mitochondrial biogenesis, improving the functioning of the antioxidant defense system, and partially reducing the level of oxidative stress. However, these changes were much less pronounced in old animals.

DOI: 10.1134/S0006297924070149

Keywords: aging, beta-hydroxybutyrate, ketogenic diet, mitochondria, mitochondrial DNA, oxidative stress

INTRODUCTION

Aging is associated with accumulation of various defects leading to cell and tissue dysfunction. Organ failure during aging is thought to be related to dysregulation of nutrient metabolism and mitochondrial function [1]. Mitochondria are major organelles responsible for the maintenance of cell energy homeostasis. Their main function is ATP production, but mitochondria are also involved in other cellular processes, such as regulation of calcium metabolism and redox status of the cell, synthesis of metabolic intermediates,

* To whom correspondence should be addressed.

generation of reactive oxygen species (ROS), and regulation of apoptosis. Mitochondrial dysfunction is one of the key factors in the pathogenesis of various diseases [2]. Due to excessive ROS production or lack of mitochondrial antioxidants [3], the damage and mutations in the mitochondrial DNA (mtDNA) can accumulate with age, impairing the functioning of mitochondrial respiratory chain and leading to a further increase in the ROS production and damage to cellular components [4].

Diet is an important factor influencing metabolism and mitochondrial functions. The effects of a diet on various aspects of metabolism are currently actively investigated in basic research and clinical studies. The ketogenic diet, which is characterized by a low content of carbohydrates, moderate protein intake, and high content of fats, has recently become increasingly popular [5]. This diet stimulates production in the liver of compounds called the ketone bodies, e.g., β-hydroxybutyrate (BHB), which are effective in preventing and treating of certain diseases, including epilepsy [6], Alzheimer's disease [7], Parkinson's disease [8], cardiovascular disorders [9], and stroke [10].

It had been previously thought that in many tissues, mitochondria use BHB as an alternative substrate for ATP production to significantly improve the energy supply to the tissue. However, there is a growing point of view that BHB is not only a metabolic substrate but also an important regulator of physiological processes that acts through various signaling mechanisms [11]. In particular, BHB significantly improves mitochondrial functions and downregulates mitochondrial Ca^{2+} levels, ROS production, and mitochondrial permeability transition pore (mPTP) activation, thus contributing to the reduction of inflammatory processes [12].

The effects of BHB as a transcriptional modulator, particularly in animals of different age, have been studied to a much lesser extent, although BHB is known to affect the level of histone acetylation [13] and, consequently, gene expression. BHB may also contribute to changes in the activity of the Nrf2/ARE [14], PGC-1α, FoxO1 [15], and NF-kB [16] signaling pathways. These signaling pathways are associated with the regulation of mitochondrial processes; some of them are considered as promising targets for geroprotection [17, 18].

The aim of this study was to analyze the effects of BHB administration for 2 weeks on the expression of genes coding for proteins involved in mitochondrial biogenesis, quality control, antioxidant defense functioning, and inflammation in major organs, such as brain, lung, heart, liver, kidney, and muscle. We assessed changes in the mtDNA copy number, amount of large-scale deletions, and extent of mtDNA damage in the organs of young and old rats to investigate the effect of BHB on the integrity of mitochondrial genome. We also analyzed the levels of oxidative stress in different tissues and the effects of aging and BHB therapy on this process.

Abbreviations: BHB, beta-hydroxybutyrate; mtDNA, mitochondrial DNA; ROS, reactive oxygen species; TBARS, thiobarbituric acid-reactive substances.

MATERIALS AND METHODS

Animals and experimental design. Experiments were carried out in young (n = 12; age, 3-4 months; body weight, 300-400 g) and old (n = 15; age, 27 months; body weight, 600-800 g) male Wistar rats. The protocols for working with the animals were approved by the Ethics Committee of the Belozersky Research Institute of Physico-Chemical Biology of the Lomonosov Moscow State University (protocol no. 006-1/1/2024). All manipulations with the animal were performed in accordance with the ARRIVE guidelines. Young and old animals were randomly divided into 2 groups: the experimental group received a subcutaneous injection of BHB at a dose of 200 mg/kg day for 14 days, and the control group received a subcutaneous injection of an equivalent volume of saline. On day 14 of BHB administration, the animals were sacrificed, and tissue samples (brain, lungs, heart, liver, kidneys, and muscles) were collected for further analysis. The last injection of BHB was performed 1 h before freezing the organs in liquid nitrogen.

Assessment of gene expression levels. RNA was isolated using ExtractRNA reagent (Evrogen, Russia); cDNA was synthesized with a REVERTA-L kit (AmpliSens, Russia). Quantitative PCR (qPCR) was performed with a CFX96™ Real-Time System thermal cycler (Bio-Rad, USA) using a qPCRmix-HS SYBR kit. Normalized gene expression levels were calculated using the formula $2^{(-\Delta\Delta Cq)}$. Gene expression results were presented as heat maps that were produced using the Bio-Rad CFX Manager software (version 2.1). For genes with statistically significant changes in expression, the results were further presented as histograms. The *Gapdh* gene was used as a reference. The primer sequences for the studied genes are shown in Table 1.

Assessment of mtDNA copy number. Relative mtDNA copy number was assessed by qPCR using a 5X qPCRmix-HS SYBR mixture (Evrogen). To amplify rat mtDNA, the following pair of primers was used: forward: 5′-CTCAAAGGACTTGGCGGTACT-3′; reverse: 5′-GCTGAATTAGCGAGAAGGGGT-3′. The obtained results were normalized to a fragment of nuclear DNA amplified with the following primers: forward: 5′-GGCTCCCTAGGCCCCTCCTG-3′; reverse: 5′-TCCCAACTCGGCCCCCAACA-3′. Reaction conditions: denaturation at 95°C for 3 min; 35 cycles of denaturation at 95°C for 10 s, primer annealing at 59°C for 30 s,

Table 1. Primer sequences for assessing gene expression in rats

Gene	Forward primer, 5′-3′	Reverse primer, 5′-3′
Gapdh	GGCTCCCTAGGCCCCTCCTG	TCCCAACTCGGCCCCCAACA
Gfap	CGAAGAAAACCGCATCACCA	CCGCATCTCCACCGTCTTTA
Gpx1	AGTCCACCGTGTATGCCTTCT	GAGACGCGACATTCTCAATGA
Gpx3	CCATTCGGCCTGGTCATTCT	CCCGGTCGAACGTACTTGAG
Il1b	GGCTTCCTTGTGCAAGTGTCT	TCTGGACAGCCCAAGTCAAG
Nfe2l2	CACATCCAGACAGACACCAGT	CTACAAATGGGAATGTCTCTGC
Nrf1	TTACTCTGCTGTGGCTGATGG	CCTCTGATGCTTGCGTCGTCT
Ppargc1a	GCACCAGAAAACAGCTCCAA	TTGCCATCCCGTAGTTCACT
Ptgs2	CTCTGCGATGCTCTTCCGAG	TCTTGTCAGAAACTCAGGCGTA
Slc6a9	AAGGGATGYTGAATGGTGCT	TAGCCCACGCTCGTCAGTA
Slc7a11	CTGGGCAKGAGAAGGTRGTTC	CCCTTGGGGGAGATGAAGATG
Sqstm1	TCCCTGTCAAGCAGTATCC	TCCTCCTTGGCTTTGTCTC
Srxn1	CCARGGYGGYGACTACTACTA	AAGGGTYGACCTCACGAGCTT
Tfam	ATCAAGACTGTGCGTGCATC	AGAACTTCACAAACCCGCAC
Tnf	ATGGGCTCCCTCTCATCAGT	GCTTGGTGGTTTGCTACGAC
Txnrd2	GATCTCTTGGTGATCGGTGGG	CGGGGAGAGGGTTCCACATA

and elongation at 72°C for 30 s. mtDNA copy number was calculated with the Bio-Rad CFX Manager software (version 2.1) using the formula $2^{(-\Delta\Delta Cq)}$.

Estimation of the amount of large-scale mtDNA deletions. Total DNA from rat tissues was isolated with a PROBA-GS kit (DNA-technology, Russia) according to the protocol recommended by the manufacturer. The relative number of large-scale deletions in rat mtDNA ($\Delta mtDNA_{4834}$) was determined by qPCR using TaqMan probes as described previously [19]. The amount of $\Delta mtDNA_{4834}$ (a region formed by the joined sequences flanking the deleted mtDNA region) was normalized to the total amount of mtDNA. To normalize the number of deletions to the number of mtDNA copies, the D-loop region was amplified using 5X qPCRmix-HS mixture (Evrogen). Reaction conditions: denaturation at 95°C for 3 min; 35 cycles of denaturation at 95°C for 10 s, primer annealing at 64°C for 15 s, and elongation at 72°C for 20 s.

Estimation of the extent of mtDNA damage. The extent of mtDNA damage was assessed by PCR of long fragments using Encyclo polymerase (Evrogen). The protocol and the primer panel for rat mtDNA were developed and optimized previously [20]. The method is based on the assumption that the presence of damage in the DNA structure (e.g., single-strand breaks, modified bases, nucleotide adducts) impairs the functioning of DNA polymerase and reduces accumulation of PCR product. Therefore, the efficiency of amplification is inversely proportional to the number of DNA molecules with a damage in the amplified DNA region. Reaction conditions: denaturation at 95°C for 3 min; 35 cycles of denaturation at 95°C for 10 s, primer annealing at 59°C for 4 min 30 s, and elongation at 72°C for 30 s. The ΔCq values for the control and experimental (damaged) long mtDNA fragments were compared with the ΔCq values for the control and experimental short fragments that were used as references.

Measurement of thiobarbituric acid reactive substances (TBARS). The content of TBARS was determined by a spectrophotometric method based on the reaction with 2-thiobarbituric acid [21] with some modifications. Rat tissues were homogenized in 1× PBS (pH 7.4) at a 1 : 10 (*w*/*v*) ratio. The homogenate (200 µl) was mixed with 20 µl of 50% trichloroacetic acid and 800 µl of TBA reagent containing 0.5% (*w*/*v*) 2-thiobarbituric acid, 0.35 M sodium hydroxide, and 1.75 M acetic acid. The samples were boiled for 1 h, cooled in an ice bath to room temperature, and centrifuged at 10,000*g* for 10 min at 4°C. The optical density of the supernatant was measured at 532 nm on an Ekros PE-5400UF spectrophotometer (Ekroskhim, Russia).

The content of TBARS was calculated using a molar extinction coefficient of 156,000 $M^{-1}cm^{-1}$ and expressed in nmol/mg total protein. The total protein content in the homogenate was determined using the Lowry method [22].

Measurement of reduced glutathione (GSH) content. The levels of GSH were determined by a spectrophotometric method based on the reaction with the Ellman's reagent [23] with some modifications. Rat tissues were homogenized in a buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM KCl, 1 mM EDTA, 0.5% Triton X-100 at a 1 : 10 (*w*/*v*) ratio. Deproteinization was carried out by adding 50% trichloroacetic acid to the homogenate at a 1 : 9 ratio, and the samples were centrifuged for 10 min at 10,000*g* at 4°C. Next, the supernatant was mixed with 0.4 M Tris-HCl (pH 8.9) containing 1 mM EDTA at a 1 : 2 (*v*/*v*) ratio, and Ellman's reagent was added to a final concentration of 0.2 mmol/liter. The samples were incubated at room temperature for 15 min and the optical density was measured at 412 nm on an Ekros PE-5400UF spectrophotometer. The GSH content was calculated using a molar extinction coefficient of 14,150 $M^{-1}cm^{-1}$ and expressed in nmol/mg total protein. The total protein content in the homogenate was determined using the Lowry method [22].

Statistical analysis. Statistical analysis was performed with the STATISTICA 12 software package (StatSoft, USA). The results are presented as mean ± standard error of the mean (SEM). The *t*-test or nonparametric Kruskal–Wallis analysis of variance were used for statistical analysis.

RESULTS

Aging has a negative effect on the cell antioxidant defense system and expression of genes involved in the regulation of mitochondrial biogenesis and quality control. The expression of genes involved in mitochondrial biogenesis, inflammation, and functioning of the quality control and antioxidant defense systems was analyzed in the organs of young and old rats (Fig. 1). Aging significantly altered expression of these genes, especially in the brain, muscles, and lungs (Fig. 1). In particular, we revealed an age-dependent decrease in the levels of mRNAs for enzymes regulating mitochondrial biogenesis (*Ppargc1a, Tfam*) in the muscles and brain (Fig. 1, a-c). Age-related changes in the muscles were associated with a decrease in the expression of *Sqstm1*, whose product is involved in protein and organelle quality control (Fig. 1d). We also revealed a significant decrease in the expression of genes associated with the antioxidant defense system (*Gpx1*, *Srxn1*, *Txnrd2*, *Slc7a11*, and *Slc6a9*) in the lungs and muscles of old rats (Fig. 1, e-l). The observed negative changes were also accompanied by upregulation of inflammatory markers, in particular, elevated expression of *Tnf*, *Ptgs2*, *Gfap*, and *Il1b*, especially in the brain and kidneys of old rats (Fig. 1, m-p).

BHB upregulates expression of genes involved in mitochondrial biogenesis and antioxidant defense system in the liver and muscles of young but not old rats. We analyzed the effect of BHB on the expression of studied genes in the organs of young and old rats and found that subcutaneous injection of BHB for 2 weeks caused the most pronounced positive effect in the liver and muscles of young rats. In particular, in the liver, BHB increased expression of genes related to mitochondrial biogenesis (*Ppargc1a*, *Nrf1*, *Tfam*) (Fig. 2, a-c) and antioxidant defense system (*Nfe2l2*, *Gpx1*, *Txnrd2*, *Slc7a11*, *Slc6a9*) (Fig. 2, d-h). In the muscles, BHB also stimulated genes involved in mitochondrial biogenesis (*Nrf1*, *Tfam*) and antioxidant defense (*Srxn1*, *Slc6a9*) (Fig. 2, i-l). However, in old rats, BHB only slightly affected expression of the analyzed genes; no upregulated expression of genes related to mitochondrial biogenesis and antioxidant defense system was observed in the liver, muscles, or other organs (Fig. 3).

Aging influences mtDNA copy number in tissues. Since aging and BHB significantly affected expression of genes encoding proteins responsible for mitochondrial biogenesis and antioxidant defense system, mtDNA copy number, which indirectly reflects the number of mitochondria, was measured in the organs of young and old rats (Fig. 4). The mtDNA copy number was significantly lower in the brain and lungs of 27-month-old rats compared to 4-month-old rats. However, in the kidneys and heart of old rats, the mtDNA copy number was increased. In other organs, the mtDNA copy number in young and old rats did not differ. Administration of BHB had no significant effect on the mtDNA copy number in either young or old rats.

Aging increases the amount of large-scale deletions and extent of mtDNA damage. It is known that aging is associated with disturbances in the protein and organelle quality control. Therefore, we assessed the level of large-scale deletions and extent of mtDNA damage in the organs of young and old rats (Figs. 5 and 6). The number of large-scale mtDNA deletions in the kidneys and heart (but not in other organs) increased with aging (Fig. 6). Thus, the extent of mtDNA damage in the kidneys of old rats was by 53% higher than in young 4-month-old rats (Fig. 5). Administration of BHB had no effect on the number of large-scale mtDNA deletions and extent of mtDNA damage in either young or old animals (Figs. 5, 6).

Effect of aging and BHB administration on the oxidative stress level in rat tissues. Since aging had a negative effect on the expression of genes related to the antioxidant defense system, while administration

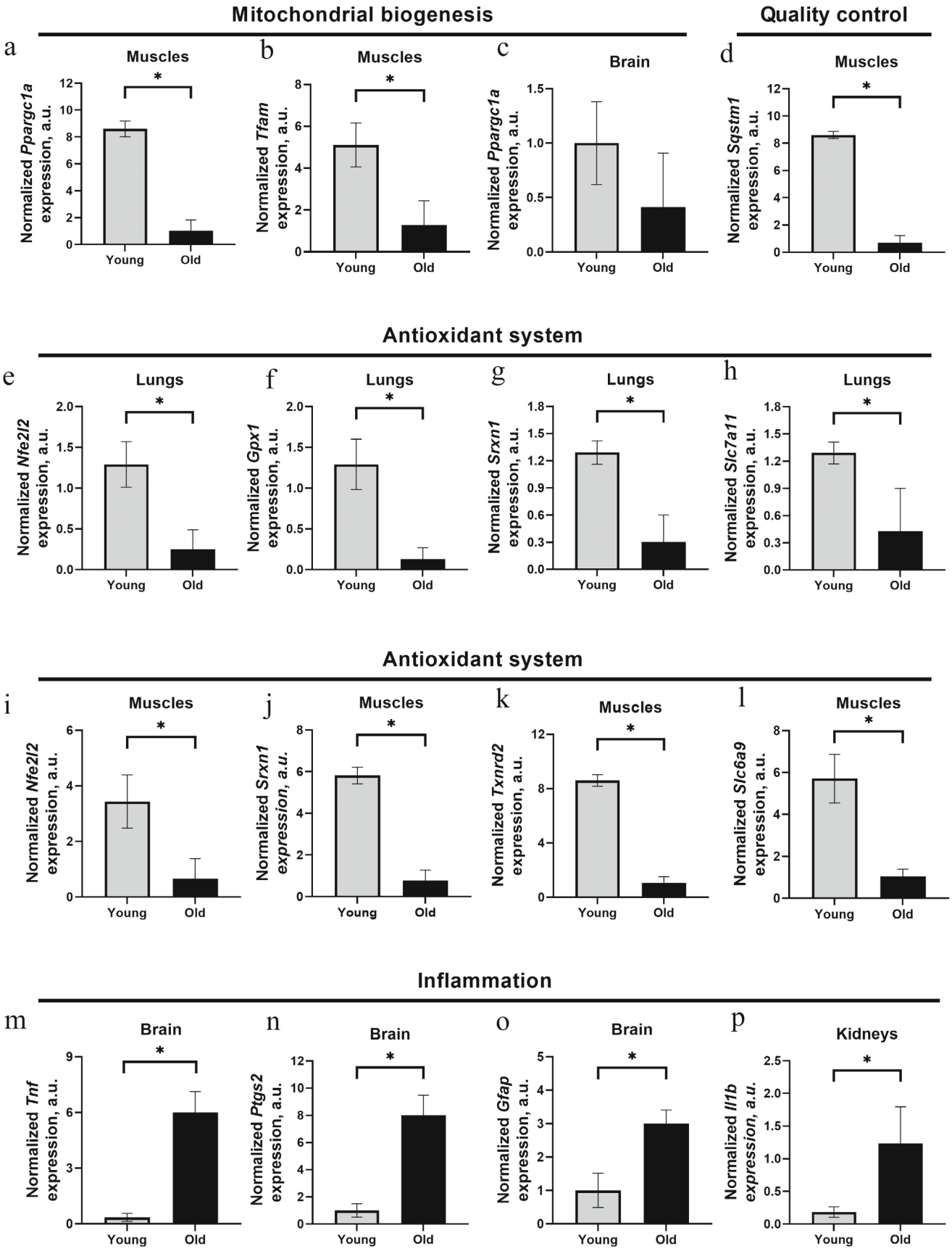

Fig. 1. Effect of aging on the expression of genes responsible for mitochondrial biogenesis (a-c), autophagy (d), antioxidant defense system (e-l), and inflammation (m-p). The data are shown as mean ± SEM; * $p < 0.05$ (*t*-test).

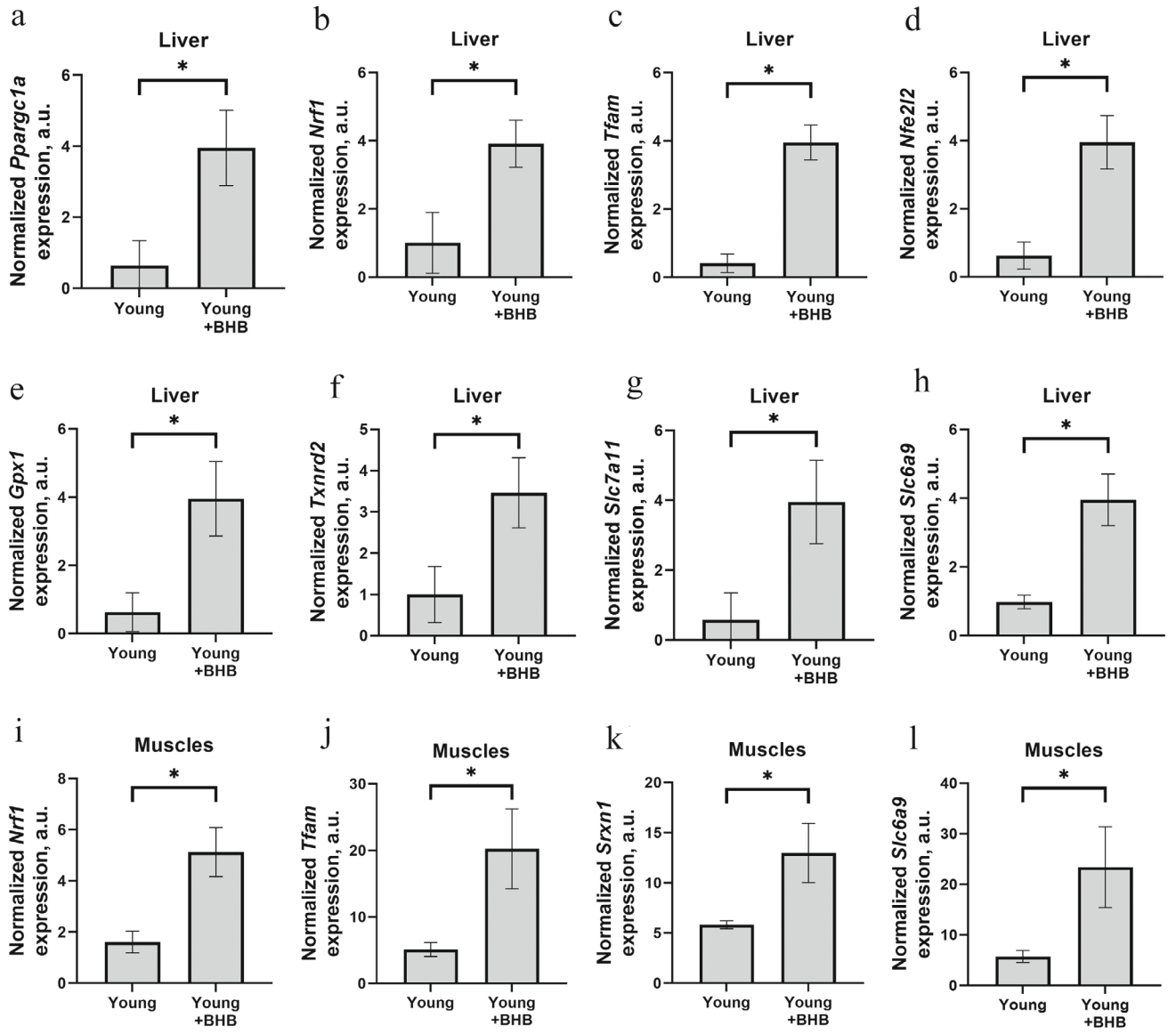

Fig. 2. Effect of subcutaneous administration of BHB on gene expression in young rats. Changes in gene expression of proteins responsible for mitochondrial biogenesis and antioxidant system are shown for liver (a-h) and muscle tissue (i-l). The data are shown as mean ± SEM; * $p < 0.05$ (*t*-test).

of BHB, on the contrary, increased expression of these genes at least in the liver and muscles of young animals, we studied the level of oxidative stress in these animals. The content of TBARS was used as a marker of oxidative stress in rat tissues. Our results showed that the TBARS concentration in the muscles of 27-month-old rats increased more than twofold compared to 4-month-old rats (Fig. 7). Similarly, the TBARS levels increased in the kidneys of old rats, while administration of BHB significantly reduced the concentration of these compounds (Fig. 7). In addition, we examined the GSH content, which decreased fourfold in the lungs of old rats compared to young ones (Fig. 8). However, administration of BHB failed to increase the GSH levels in the organs of either young or old rats.

DISCUSSION

Most researchers define aging as an irreversible complex process involving epigenetic changes [24], loss of protein homeostasis [25], metabolic disorders [26], and mitochondrial dysfunction [27]. According to the free radical theory, aging is accompanied by an imbalance between the ROS production and degradation in the mitochondria, which causes oxidative damage to various cellular macromolecules and organelles. Mitochondrial damage leads to an even greater disruption in the regulation of redox homeostasis and development of a vicious cycle promoting oxidative stress [28].

Aging can be manifested differently in different tissues. In particular, it was shown that the age-dependent

changes in the patterns of gene expression differ depending on the organ [29]. In this study, we found that aging most strongly affected expression of particular genes (including those involved in mitochondrial biogenesis, quality control, and antioxidant defense system) in the muscles, brain, and lungs of rats (Figs. 1 and 3). Negative changes that occur during aging have been associated with an increased oxidative stress and expression of proinflammatory cytokines in some organs (Figs. 7 and 8).

The hallmarks of aging include changes in the number and functional activity of mitochondria in the tissues, which can directly affect the energy supply of cells [30]. We showed that the age-related alterations in the mtDNA copy number (which indirectly indicates the number of mitochondria) varied from organ to organ. In the brain and lungs, for example, the mtDNA copy number decreased significantly (Fig. 4), which was accompanied by a decrease in the expression of genes associated with mitochondrial biogenesis (Figs. 1 and 3). At the same time, the mtDNA copy number in the kidneys and heart increased with age (Fig. 4), which may be an adaptive response of these organs to an elevated extent of mtDNA damage and growing number of large-scale deletions. Violation of the structural integrity of mitochondrial genome can lead to the disruption of cellular energy metabolism and, as a consequence, activation of signaling pathways aimed at promotion of mitochondrial biogenesis [31]. Indeed, we observed an age-related increase in the expression of *Tfam* and *Nrf1* genes in the heart and kidneys (Fig. 3).

In order to maintain an efficient energy metabolism, it is essential to maintain the stability of mtDNA in the cells. The mitochondrial genome does not have sufficiently effective repair system for particular types of DNA damage, making it more susceptible to external and internal stressors compared to the nuclear genome [32]. The frequency of mtDNA deletions

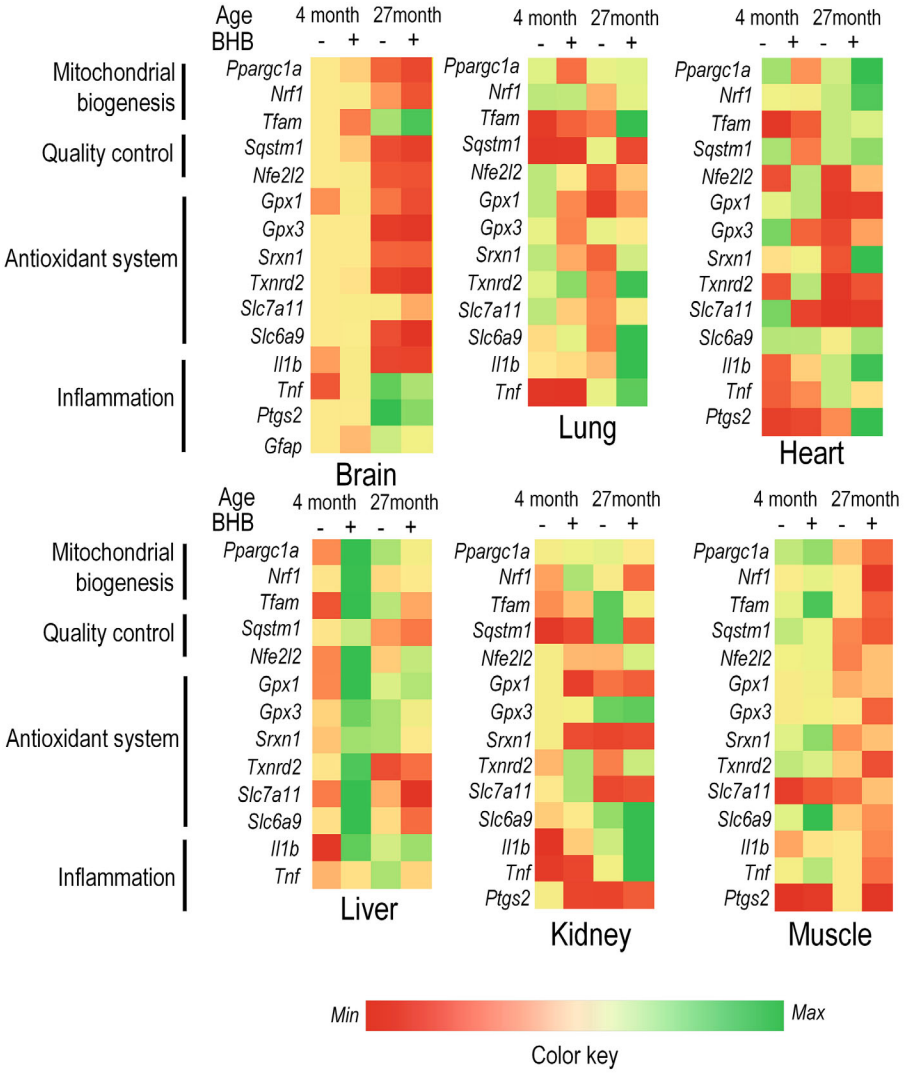

Fig. 3. Heat maps showing the effects of aging and BHB treatment on gene expression in the brain, lungs, heart, liver, kidneys, and muscles of rats.

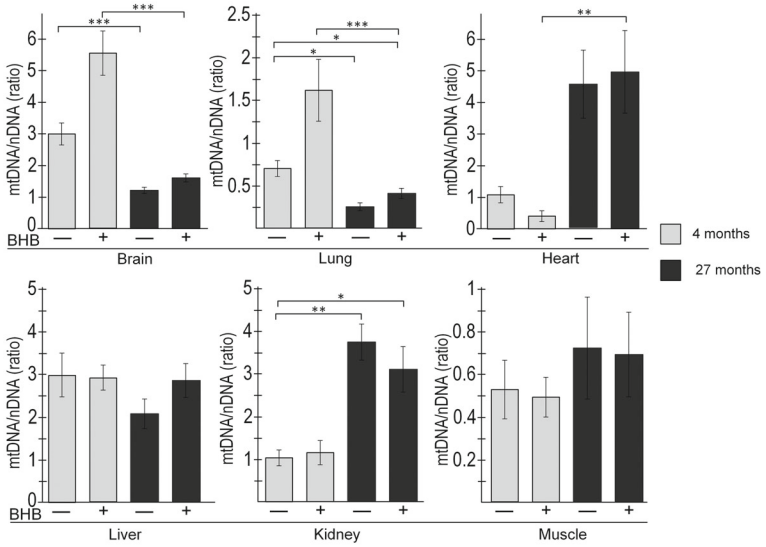

Fig. 4. Effect of subcutaneous BHB injection on the mtDNA copy number in the tissues of rats of different age. The data are presented as mean ± SEM; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Kruskal–Wallis test).

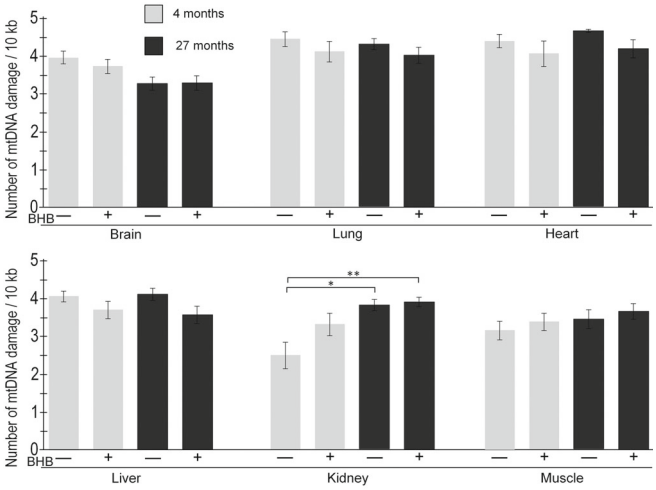

Fig. 5. Effect of subcutaneous BHB injections on the extent of mtDNA damage in the tissues of young and old rats. The data are shown as mean ± SEM; * $p < 0.05$, ** $p < 0.01$ in comparison with 4-month-old rats (Kruskal–Wallis test).

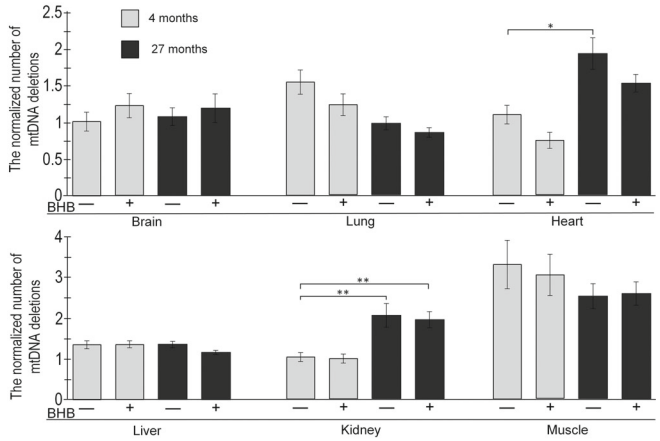

Fig. 6. Effect of subcutaneous BHB injections on the amount of large-scale deletions in mtDNA from the tissues of young and old rats. The data are shown as mean ± SEM; * $p < 0.05$, ** $p < 0.01$ in comparison to 4-month-old rats (Kruskal–Wallis test).

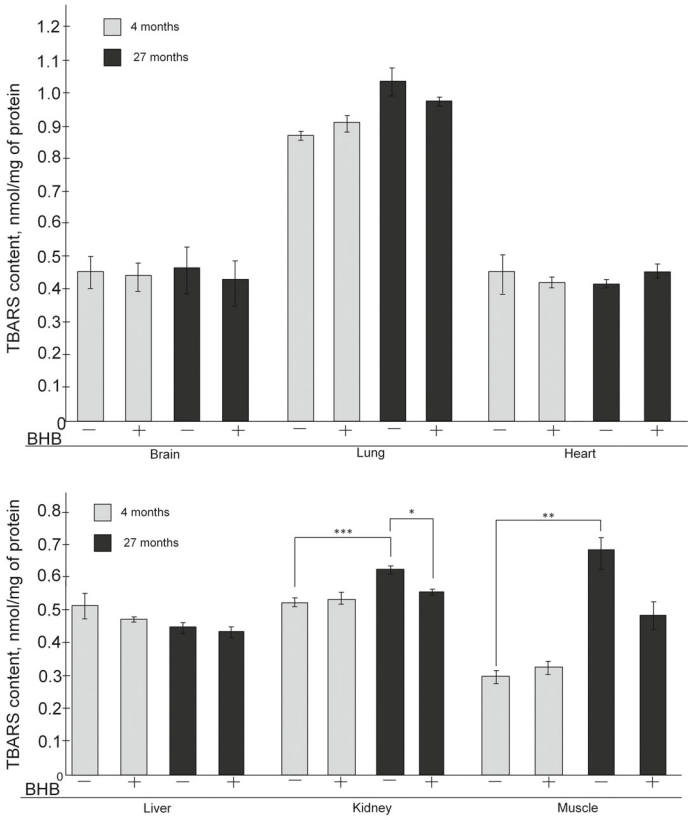

Fig. 7. Effect of subcutaneous injections of BHB on the TBARS content in the tissues of young and old rats. The data are expressed as mean ± SEM. * p <0.05, ** p < 0.01, and *** p < 0.001 compared to 4-month-old rats (Kruskal–Wallis test).

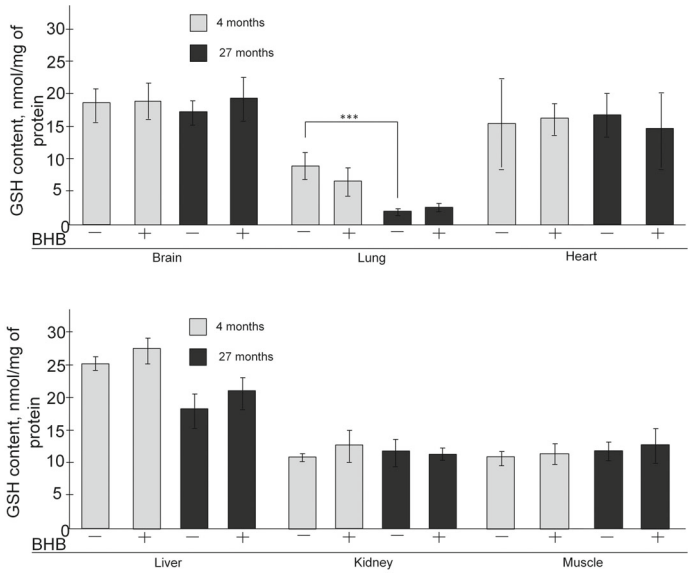

Fig. 8. Effect of subcutaneous injections of BHB on the GSH content in the tissues of young and old rats. The data in a group are expressed as mean ± SEM; *** p < 0.001 in comparison to 4-month-old rats (Kruskal–Wallis test).

increases with age, and these changes are most common in the tissues with high energy consumption [33]. An increase in the number of large-scale deletions can lead to age-related organ dysfunction due to the loss of important coding sequences, resulting in defective respiratory chain assembly [34]. In this study, we showed that the organ most susceptible to such type of damage is kidney, where most large-scale deletions and mtDNA damage accumulate with age (Figs. 5, 6). The kidney is a mitochondria-rich organ, and mutations in mtDNA

and its damage are important contributors to the development of chronic and acute kidney diseases [35].

The increase in the amount of large-scale mtDNA deletions with age was more pronounced that the increase in the extent of mtDNA damage (Figs. 5 and 6), which could be due to the fact that the long-range PCR used in our work allowed us to detect particular types of mtDNA damage, such as chain breaks, base modifications, and apurinic sites [36]. It is known that the modified nitrogenous bases and nucleotides in mtDNA can be repaired, as mitochondria have base and nucleotide excision repair systems (BER and NER, respectively), while the systems for the repair of DNA breaks, especially, double-strand breaks, are poorly represented in the mitochondria [37]. Basically, most mtDNA molecules with double-strand breaks are eliminated. However, double-strand breaks are one of the main causes of mtDNA deletions [38]. Molecules containing deletions can replicate and accumulate throughout the lifetime and can even be inherited via the maternal line [39], whereas damaged molecules, which we detected by long-fragment PCR, are either repaired or degraded. This could explain why point mutations in mtDNA accumulate with age to a lesser extent than large-scale deletions.

One of the approaches to treating age-related diseases is modulation of cell metabolism through various diets or their pharmacological mimetics. In particular, the use of ketone bodies, whose levels increase in the case of ketogenic diet or caloric restriction, has been proposed as a component of geroprotective therapy [40]. It has already been shown that administration of exogenous BHB leads to global changes in gene transcription, including changes in the expression of genes encoding factors determining cell resistance to oxidative stress [41].

In our study, we demonstrated that BHB produced different effects on gene expression in different organs, and these effects varied significantly between the young and old rats. Among the organs analyzed, the widest range of changes in gene expression upon BHB treatment was observed in the liver of young rats (Fig. 3). Although liver synthesizes ketone bodies, it is unable to metabolize them [42]. BHB upregulated expression of genes related to mitochondrial biogenesis in the liver, as well as those coding for enzymes of the cell antioxidant defense system (Fig. 3). Mitochondrial biogenesis and mitophagy contribute to the maintenance of the appropriate number of mitochondria and their quality in the cells [43, 44]. Therefore, activation of mitochondrial biogenesis should be accompanied by the corresponding removal of damaged or poorly functioning organelles [45].

The pronounced effects of BHB in the liver may be due to the fact that liver is the main source of endogenous BHB [46] and that the highest concentrations of ketone bodies during administration of exogenous BHB can be achieved in the liver. The effect of BHB in the liver may be mediated by β-hydroxybutyrylation of histones [47], which affects gene expression, as well as through signaling via the HCAR2 receptor. In particular, BHB was previously shown to inhibit NLRP3 inflammasome-mediated inflammation and also to display a hepatoprotective effect by acting on HCAR2 [48]. Through the HCAR2 signaling, BHB activates AMPK, which may potentially improve metabolic functions impaired in hepatic steatosis [49]. Another possible mechanism is inhibition of class I histone deacetylases (HDACs). Class I HDACs deacetylate lysine residues in histones and non-histone proteins and regulate genes involved in gluconeogenesis, mitochondrial function, and protection against oxidative stress [50]. Inhibition of HDACs blocks proteasomal degradation of Nrf2, thereby facilitating its binding to the promoter regions of genes responsible for cell antioxidant defense [51]. BHB was also found to inhibit glycogen synthase kinase-3β (GSK3β), which is a negative regulator of Nrf2 [52]. We have previously shown that the treatment of focal ischemic stroke with BHB contributed to the upregulation of expression of several important antioxidant defense genes via an Nrf2-dependent mechanism [53]. Therefore, the increase in the expression of antioxidant defense genes as a result of ketone body therapy observed in our study might be related to the activation of the Nrf2/ARE signaling pathway. This is also indicated by the upregulated *Nfe2l2* expression in the liver and heart of rats receiving BHB injections (Figs. 2 and 3), as it is known that the *Nfe2l2* promoter contains ARE sequences [54], which provides self-amplification of its expression.

Beside affecting gene expression, the administration of BHB led to a drop in the levels of lipid peroxidation products, which were otherwise increased in the kidneys and muscles of old animals (Fig. 7). These results are consistent with the previous data showing that administration of exogenous BHB protected kidneys from the oxidative stress induced by cisplatin [55] and that the ketogenic diet led to positive changes in the muscles [56]. However, BHB had no significant effect on the TBARS content in other organs of young and old animals, although it significantly affected expression of genes related to the cellular antioxidant defense systems in the liver and muscles of young rats (Figs. 2 and 3).

CONCLUSION

In this study, we showed that the BHB therapy affected differently various organs in the animals of different age. BHB strongly altered expression of genes involved in mitochondrial biogenesis and cellular

antioxidant defense primarily in the liver and muscles of young rats. The stimulation of mitochondrial biogenesis might trigger mitochondrial renewal, leading to the improved functioning of mitochondria and decreased levels of oxidative stress. However, despite these positive effects, no significant influence of BHB on the mtDNA integrity was observed in either young or old animals. We suggest that achieving a more pronounced effect on the mtDNA integrity, oxidative stress, and inflammation requires a longer therapy with BHB. The data obtained in this study expands the understanding of therapeutic effects of ketone bodies, including their potential use in the treatment of age-related diseases.

Contributions. E.Y.P. developed the concept and supervised the study; V.V.N., P.I.B., N.A.S., I.S.S., D.S.S., A.A.B., and N.V.A. conducted the experiments; N.V.A., A.P.G., and E.Y.P. discussed the results; V.V.N., N.V.A., and A.P.G. wrote the text of the article; E.Y.P. edited the manuscript.

Funding. This research was supported by the Russian Science Foundation (project no. 21-75-30009).

Ethics declarations. The animal protocols were reviewed and approved by the Animal Ethics Committee of the Belozersky Institute of Physico-Chemical Biology Lomonosov Moscow State University (Protocol № 006-1/1/2024). All procedures were performed in accordance with the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines. The authors of this work declare that they have no conflicts of interest.

REFERENCES

1. Son, J. M., and Lee, C. (2019) Mitochondria: multifaceted regulators of aging, *BMB Rep.*, **52**, 13-23, https://doi.org/10.5483/BMBRep.2019.52.1.300.
2. Harrington, J. S., Ryter, S. W., Plataki, M., Price, D. R., and Choi, A. M. K. (2023) Mitochondria in health, disease, and aging, *Physiol. Rev.*, **103**, 2349-2422, https://doi.org/10.1152/physrev.00058.2021.
3. Whitehall, J. C., Smith, A. L. M., and Greaves, L. C. (2023) Mitochondrial DNA mutations and ageing, *Subcell. Biochem.*, **102**, 77-98, https://doi.org/10.1007/978-3-031-21410-3_4.
4. Nissanka, N., and Moraes, C. T. (2018) Mitochondrial DNA damage and reactive oxygen species in neurodegenerative disease, *FEBS Lett.*, **592**, 728-742, https://doi.org/10.1002/1873-3468.12956.
5. Ivannikova, E. V., Altashina, M. A., and Troshina, E. A. (2021) The ketogenic diet: history, mechanism of action, indications and contraindications [in Russian], *Probl. Endokrinol. (Moscow)*, **68**, 49-72, https://doi.org/10.14341/probl12724.
6. Kumar, A., Kumari, S., and Singh, D. (2022) Insights into the cellular interactions and molecular mechanisms of ketogenic diet for comprehensive management of epilepsy, *Curr. Neuropharmacol.*, **20**, 2034-2049, https://doi.org/10.2174/1570159X20666220420130109.
7. Shippy, D. C., Wilhelm, C., Viharkumar, P. A., Raife, T. J., and Ulland, T. K. (2020) β-Hydroxybutyrate inhibits inflammasome activation to attenuate Alzheimer's disease pathology, *J. Neuroinflammation*, **17**, 280, https://doi.org/10.1186/s12974-020-01948-5.
8. Norwitz, N. G., Hu, M. T., and Clarke, K. (2019) The mechanisms by which the ketone body D-β-hydroxybutyrate may improve the multiple cellular pathologies of Parkinson's disease, *Front. Nutr.*, **6**, 63, https://doi.org/10.3389/fnut.2019.00063.
9. Wei, S., Binbin, L., Yuan, W., Zhong, Z., Donghai, L., and Caihua, H. (2022) β-Hydroxybutyrate in cardiovascular diseases: a minor metabolite of great expectations, *Front. Mol. Biosci.*, **13**, 823602, https://doi.org/10.3389/fmolb.2022.823602.
10. Li, Y., Zhang, X., Ma, A., and Kang, Y. (2021) Rational application of β-hydroxybutyrate attenuates ischemic stroke by suppressing oxidative stress and mitochondrial-dependent apoptosis via activation of the Erk/CREB/eNOS pathway, *ACS Chem. Neurosci.*, **12**, 1219-1227, https://doi.org/10.1021/acschemneuro.1c00046.
11. Rojas-Morales, P., Pedraza-Chaverri, J., and Tapia, E. (2020) Ketone bodies, stress response, and redox homeostasis, *Redox Biol.*, **29**, 101395, https://doi.org/10.1016/j.redox.2019.101395.
12. Jin, L. W., Lucente, J. D., Mendiola, U. R., Suthprasertporn, N., Tomilov, A., Cortopassi, G., Kim, K., Ramsey, J. J., and Maezawa, I. (2023) The ketone body β-hydroxybutyrate shifts microglial metabolism and suppresses amyloid-β oligomer-induced inflammation in human microglia, *FASEB J.*, **37**, e23261, https://doi.org/10.1096/fj.202301254R.
13. Newman, J. C., and Verdin, E. (2017) β-Hydroxybutyrate: a signaling metabolite, *Annu. Rev. Nutr.*, **37**, 51-76, https://doi.org/10.1146/annurev-nutr-071816-064916.
14. Goshtasbi, H., Pakchin, P. S., Movafeghi, A., Barar, J., Castejon, A. M., Omidian, H., and Omidi, Y. (2022) Impacts of oxidants and antioxidants on the emergence and progression of Alzheimer's disease, *Neurochem. Int.*, **153**, 105268, https://doi.org/10.1016/j.neuint.2021.105268.
15. Kim, D. H., Park, M. H., Ha, S., Bang, E. J., Lee, Y., Lee, A. K., Lee, J., Yu, B. P., and Chung, H. Y. (2019) Anti-inflammatory action of β-hydroxybutyrate via modulation of PGC-1α and FoxO1, mimicking calorie restriction, *Aging (Albany NY)*, **11**, 1283-1304, https://doi.org/10.18632/aging.101838.
16. Shi, X., Li, X., Li, D., Li, Y., Song, Y., Deng, Q., Wang, J., Zhang, Y., Ding, H., Yin, L., Zhang, Y., Wang, Z., Li, X., and Liu, G. (2014) β-Hydroxybutyrate activates the NF-κB signaling pathway to promote the

expression of pro-inflammatory factors in calf hepatocytes, *Cell Physiol. Biochem.*, **33**, 920-932, https://doi.org/10.1159/000358664.

17. Martins, R., Lithgow, G. J., and Link, W. (2016) Long live FOXO: unraveling the role of FOXO proteins in aging and longevity, *Aging Cell.*, **15**, 196-207, https://doi.org/10.1111/acel.12427.
18. Gureev, A. P., Shaforostova, E. A., and Popov, V. N. (2019) Regulation of mitochondrial biogenesis as a way for active longevity: interaction between the Nrf2 and PGC-1α signaling pathways, *Front. Genet.*, **14**, 435, https://doi.org/10.3389/fgene.2019.00435.
19. Loshchenova, P. S., Sinitsyna, O. I., Fedoseeva, L. A., Stefanova, N. A., and Kolosova, N. G. (2015) Influence of antioxidant SkQ1 on accumulation of mitochondrial DNA deletions in the hippocampus of senescence-accelerated OXYS rats, *Biochemistry (Moscow)*, **80**, 596-603, https://doi.org/10.1134/S0006297915050120.
20. Gureev, A. P., Andrianova, N. V., Pevzner, I. B., Zorova, L. D., Chernyshova, E. V., Sadovnikova, I. S., Chistyakov, D. V., Popkov, V. A., Semenovich, D. S., Babenko, V. A., Silachev, D. N., Zorov, D. B., Plotnikov, E. Y., and Popov, V. N. (2022) Dietary restriction modulates mitochondrial DNA damage and oxylipin profile in aged rats, *FEBS J.*, **289**, 5697-5713, https://doi.org/10.1111/febs.16451.
21. Ohkawa, H., Ohishi, N., and Yagi, K. (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction, *Anal. Biochem.*, **95**, 351-358, https://doi.org/10.1016/0003-2697(79)90738-3.
22. Hartree, E. F. (1972) Determination of protein: a modification of the Lowry method that gives a linear photometric response, *Anal. Biochem.*, **48**, 422-427, https://doi.org/10.1016/0003-2697(72)90094-2.
23. Patsoukis, N., and Georgiou, C. D. (2004) Determination of the thiol redox state of organisms: new oxidative stress indicators, *Anal. Bioanal. Chem.*, **378**, 1783-1792, https://doi.org/10.1007/s00216-004-2525-1.
24. Pal, S., and Tyler, J. K. (2016) Epigenetics and aging, *Sci. Adv.*, **2**, e1600584, https://doi.org/10.1126/sciadv.1600584.
25. Hoppe, T., and Cohen, E. (2020) Organismal protein homeostasis mechanisms, *Genetics*, **215**, 889-901, https://doi.org/10.1534/genetics.120.301283.
26. Gregory, J. W. (2009) Metabolic disorders, *Endocr. Dev.*, **15**, 59-76, https://doi.org/10.1159/000207610.
27. Lin, M. T., and Beal, M. F. (2006) Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases, *Nature*, **443**, 787-795, https://doi.org/10.1038/nature05292.
28. Zorov, D. B., Isaev, N. K., Plotnikov, E. Y., Zorova, L. D., Stelmashook, E. V., Vasileva, A. K., Arkhangelskaya, A. A., and Khrjapenkova, T. G. (2007) The mitochondrion as Janus Bifrons, *Biochemistry (Moscow)*, **72**, 1115-1126, https://doi.org/10.1134/s0006297907100094.
29. Schaum, N., Lehallier, B., Hahn, O., Pálovics, R., Hosseinzadeh, S., Lee, S. E., Sit, R., Lee, D. P., Losada, P. M., Zardeneta, M. E., Fehlmann, T., and Webber, J. T. (2020) Ageing hallmarks exhibit organ-specific temporal signatures, *Nature*, **583**, 596-602, https://doi.org/10.1038/s41586-020-2499-y.
30. Lima, T., Li, T. Y., Mottis, A., and Auwerx, J. (2022) Pleiotropic effects of mitochondria in aging, *Nat. Aging.*, **2**, 199-213, https://doi.org/10.1038/s43587-022-00191-2.
31. Masuyama, M., Iida, R., Takatsuka, H., Yasuda, T., and Matsuki, T. (2005) Quantitative change in mitochondrial DNA content in various mouse tissues during aging, *Biochim. Biophys. Acta.*, **1723**, 302-308, https://doi.org/10.1016/j.bbagen.2005.03.001.
32. Sharma, P., and Sampath, H. (2019) Mitochondrial DNA integrity: role in health and disease, *Cells*, **8**, 100, https://doi.org/10.3390/cells8020100.
33. Gadaleta, M. N., Rainaldi, G., Lezza, A. M., Milella, F., Fracasso, F., and Cantatore, P. (1992) Mitochondrial DNA copy number and mitochondrial DNA deletion in adult and senescent rats, *Mutat. Res.*, **275**, 181-193, https://doi.org/10.1016/0921-8734(92)90022-h.
34. Bank, C., Soulimane, T., Schröder, J. M., Buse, G., and Zanssen, S. (2000) Multiple deletions of mtDNA remove the light strand origin of replication, *Biochem. Biophys. Res. Commun.*, **279**, 595-601, https://doi.org/10.1006/bbrc.2000.3951.
35. Feng, J., Chen, Z., Liang, W., Wei, Z., and Ding, G. (2022) Roles of mitochondrial DNA damage in kidney diseases: a new biomarker, *Int. J. Mol. Sci.*, **23**, 15166, https://doi.org/10.3390/ijms232315166.
36. Edwards, J. G. (2009) Quantification of mitochondrial DNA (mtDNA) damage and error rates by real-time QPCR, *Mitochondrion*, **9**, 31-35, https://doi.org/10.1016/j.mito.2008.11.004.
37. Zinovkina, L. A. (2018) Mechanisms of mitochondrial DNA repair in mammals, *Biochemistry (Moscow)*, **83**, 233-249, https://doi.org/10.1134/S0006297918030045.
38. Nissanka, N., Minczuk, M., and Moraes, C. T. (2019) Mechanisms of mitochondrial DNA deletion formation, *Trends Genet.*, **35**, 235-244, https://doi.org/10.1016/j.tig.2019.01.001.
39. Yan, C., Duanmu, X., Zeng, L., Liu, B., and Song, Z. (2019) Mitochondrial DNA: distribution, mutations, and elimination, *Cells*, **8**, 379, https://doi.org/10.3390/cells8040379.
40. Wang, L., Chen, P., and Xiao, W. (2021) β-Hydroxybutyrate as an anti-aging metabolite, *Nutrients*, **13**, 3420, https://doi.org/10.3390/nu13103420.
41. Shimazu, T., Hirschey, M. D., Newman, J., He, W., Shirakawa, K., Moan, N. L., Grueter, C. A., Lim, H., Saunders, L. R., Stevens, R. D., Newgard, C. B., Farese, R. V., Cabo, R., Ulrich, S., Akassoglou, K., and Verdin, E. (2013) Suppression of oxidative stress

by β-hydroxybutyrate, an endogenous histone deacetylase inhibitor, *Science*, **339**, 211-214, https://doi.org/10.1126/science.1227166.

42. Makievskaya, C. I., Popkov, V. A., Andrianova, N. V., Liao, X., Zorov, D. B., and Plotnikov, E. Y. (2023) Ketogenic diet and ketone bodies against ischemic injury: targets, mechanisms, and therapeutic potential, *Int. J. Mol. Sci.*, **24**, 2576, https://doi.org/10.3390/ijms24032576.
43. Jornayvaz, F. R., and Shulman, G. I. (2010) Regulation of mitochondrial biogenesis, *Essays Biochem.*, **47**, 69-84, https://doi.org/10.1042/bse0470069.
44. Lu, Y., Li, Z., Zhang, S., Zhang, T., Liu, Y., and Zhang, L. (2023) Cellular mitophagy: Mechanism, roles in diseases and small molecule pharmacological regulation, *Theranostics*, **13**, 736-766, https://doi.org/10.7150/thno.79876.
45. Ashrafi, G., and Schwarz, T. L. (2013) The pathways of mitophagy for quality control and clearance of mitochondria, *Cell Death Differ.*, **20**, 31-42, https://doi.org/10.1038/cdd.2012.81.
46. Han, Y., Ramprasath, T., and Zou, M. (2020) β-hydroxybutyrate and its metabolic effects on age-associated pathology, *Exp. Mol. Med.*, **52**, 548-555, https://doi.org/10.1038/s12276-020-0415-z.
47. Koronowski, K. B., Greco, C. M., Huang, H., Kim, J., Fribourgh, J. L., Crosby, P., Mathur, L., Ren, X., Partch, C. L., Jang, C., Qiao, F., Zhao, Y., and Sassone-Corsi, P. (2021) Ketogenesis impact on liver metabolism revealed by proteomics of lysine β-hydroxybutyrylation, *Cell Rep.*, **36**, 109487, https://doi.org/10.1016/j.celrep.2021.109487.
48. Pan, A., Sun, X., Huang, F., Liu, J., Cai, Y., and Wu, X. (2022) The mitochondrial β-oxidation enzyme HADHA restrains hepatic glucagon response by promoting β-hydroxybutyrate production, *Nat. Commun.*, **13**, 386, https://doi.org/10.1038/s41467-022-28044-x.
49. Lee, A. K., Kim, D. H., Bang, E., Choi, Y. J., and Chung, H. Y. (2020) β-hydroxybutyrate suppresses lipid accumulation in aged liver through GPR109A-mediated signaling, *Aging Dis.*, **11**, 777-790, https://doi.org/10.14336/AD.2019.0926.
50. Newman, J. C., and Verdin, E. (2014) β-Hydroxybutyrate: much more than a metabolite, *Diabetes Res. Clin. Pract.*, **106**, 173-181, https://doi.org/10.1016/j.diabres.2014.08.009.
51. Komaravelli, N., Tian, B., Ivanciuc, T., Mautemps, N., Brasier, A. R., Garofalo, R. P., and Casola, A. (2015) Respiratory syncytial virus infection down-regulates antioxidant enzyme expression by triggering deacetylation-proteasomal degradation of Nrf2, *Free Radic. Biol. Med.*, **88**, 391-403, https://doi.org/10.1016/j.freeradbiomed.2015.05.043.
52. Fang, Y., Chen, B., Gong, A. Y., Malhotra, D. K., Gupta, R., Dworkin, L. D., and Gong, R. (2021) The ketone body β-hydroxybutyrate mitigates the senescence response of glomerular podocytes to diabetic insults, *Kidney Int.*, **100**, 1037-1053, https://doi.org/10.1016/j.kint.2021.06.031.
53. Gureev, A. P., Sadovnikova, I. S., Chernyshova, E. V., Tsvetkova, A. D., Babenkova, P. I., Nesterova, V. V., Krutskikh, E. P., Volodina, D. E., Samoylova, N. A., Andrianova, N. V., Silachev, D. N., and Plotnikov, E. Y. (2024) Beta-hydroxybutyrate mitigates sensorimotor and cognitive impairments in a photothrombosis-induced ischemic stroke in mice, *Int. J. Mol. Sci.*, **25**, 5710, https://doi.org/10.3390/ijms25115710.
54. Kwak, M., Itoh, K., Yamamoto, M., and Kensler, T. W. (2002) Enhanced expression of the transcription factor Nrf2 by cancer chemopreventive agents: role of antioxidant response element-like sequences in the nrf2 promoter, *Mol. Cell. Biol.*, **22**, 2883-2892, https://doi.org/10.1128/MCB.22.9.2883-2892.2002.
55. Luo, S., Yang, M., Han, Y., Zhao, H., Jiang, N., Li, L., Chen, W., Li, C., Yang, J., Liu, Y., Liu, C., Zhao, C., and Sun, L. (2022) β-Hydroxybutyrate against Cisplatin-Induced acute kidney injury via inhibiting NLRP3 inflammasome and oxidative stress, *Int. Immunopharmacol.*, **111**, 109101, https://doi.org/10.1016/j.intimp.2022.109101.
56. Huang, T., Linden, M. A., Fuller, S. E., Goldsmith, F. R., Simon, J., Batdorf, H. M., Scott, M. C., Essajee, N. M., Brown, J. M., and Noland, R. C. (2021) Combined effects of a ketogenic diet and exercise training alter mitochondrial and peroxisomal substrate oxidative capacity in skeletal muscle, *Am. J. Physiol. Endocrinol. Metab.*, **320**, E1053-E1067, https://doi.org/10.1152/ajpendo.00410.2020.