INCREASED PHYSICAL ACTIVITY UNDER CONDITIONS OF NORMOXIA CAUSES IDIOPATHIC CACHEXIA IN *HETEROCEPHALUS GLABER*

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Enrichment of habitat of the captive rodents *Heterocephalus glaber* (naked mole rats) allowing them to implement the innate behavioral pattern of digging through hard soil somehow led to the emergence of unusual animals showing signs of cachexia in the colony; these differed from other animals by the reduced body mass index associated with subcutaneous fat reduction. Furthermore, the animals itself showed aggressive eating behavior, but showed no weight gain even after stopping digging due to detachment of the camera with soil. The study aimed to clarify the pathogenetic mechanism underlying the reported phenomenon. For that animals showing signs of cachexia (one female and two males aged 4–5 years) were withdrawn from the colony, along with the animals showing no such signs (two females and one male aged 4–5 years) as controls. Histologic assessment of tissues revealed cardiac hypertrophy and hyperlipofuscinosis of the liver. Cardiac hypertrophy was also suggested by the results of the animal heart microRNA sequencing bioinformatics analysis that revealed elevated levels of microRNA responsible for the increased cell division activity and reduced apoptotic activity in the heart. These data suggest that the animals living in the habitat with the increased oxygen content (21% vs. 8% in the natural habitat, underground) experienced severe oxidative stress during physical activity, which resulted in dysfunction of body's regulatory systems, increased metabolism at rest, cardiovascular system overload, and damage to organs and tissues. Thus, naked mole rats can have normal physical activity only under conditions of low oxygen content.

Keywords: naked mole rat, cachexia, physical burden, oxidative stress, lipofuscinosis, basal metabolic rate, hyperoxia

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ПОВЫШЕННАЯ ФИЗИЧЕСКАЯ НАГРУЗКА В УСЛОВИЯХ НОРМОКСИИ ВЫЗЫВАЕТ ИДИОПАТИЧЕСКУЮ КАХЕКСИЮ У *HETEROCEPHALUS GLABER*

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Обогащение среды обитания живущих в неволе грызунов *Heterocephalus glaber* (голых землекопов), позволившее им реализовать врожденный поведенческий паттерн рытья плотного грунта, неизвестным образом привело к появлению в колонии необычных животных с признаками кахексии, отличавшихся от остальных животных сниженным индексом массы тела на фоне уменьшения доли подкожного жира. Сами животные демонстрировали при этом агрессивное пищевое поведение, но не набирали веса даже после прекращения рытья при отсоединении камеры с грунтом. Целью работы было выяснить патогенетический механизм наблюдаемого явления. Для этого из колонии изъяли животных с признаками кахексии (одна самка и два самца возрастом 4–5 лет), а также животных, не имеющих данных признаков (две самки и один самец возрастом 4–5 лет) в качестве контрольных. При гистологическом анализе тканей были выявлены гипертрофия сердца и гиперлипофусциноз печени. На гипетрофию сердца также указывали результаты биоинформатического анализа секвенирования микроРНК сердца животных, который показал повышенный уровень микроРНК, ответственных за повышение активности деления клеток, и снижение активности апоптоза в сердце. Эти данные свидетельствуют о том, что животные, находясь в среде обитания с повышенным для них содержанием кислорода (21% против 8% в естественной среде обитания под землей), при выполнении физических нагрузок испытали сильный окислительный стресс, что привело к нарушению работы регуляторных систем организма, подъему метаболизма в покое, перегрузке работы сердечно-сосудистой системы и повреждению органов и тканей. Таким образом, голые землекопы могут вести нормальную для них физическую активность только в условиях низкого содержания кислорода.

Ключевые слова: голый землекоп, кахексия, физическая нагрузка, окислительный стресс, липофусциноз, метаболизм в состоянии покоя, гипероксия

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Naked mole rats (Heterocephalus glaber), the subterranean rodents inhabiting the Horn of Africa (Somali, Ethiopia), are incredibly interesting to study. One of their distinguishing features is eusociality, while the other is abnormally high life expectancy. Thus, naked mole rats having the relatively low body weight (30–80 g) can live up to 37 years under laboratory conditions [1], while the rodent with the same weight, such as house mouse (Mus musculus), lives up to 3 years under laboratory conditions and up to 1.5 years in the wild [2].

Creating housing conditions for laboratory animals similar to that in the animals' natural range is an essential component of the studies using animals for assessment of various biological processes. When dealing with naked mole rats, it is necessary to maintain high temperature and humidity. However, creating imitation of the underground labyrinth, which naked mole rats would inhabit while staying at the laboratory, is the most time-consuming process related to the naked mole rat housing [3]. The labyrinth itself consists of cylinders and connecting tunnels made of acrylic glass. However, such a housing system does not allow the animals to fully demonstrate their physical activity, since the cylinders are partially filled with the substrate, the animals could burrow. It is well known that low physical activity sometimes results in alteration of the skeletal muscle structure, which can lead to incorrect interpretation of the research results when comparing with other model objects [4]. In this regard, the laboratory staff decided to install a supplementary compartment filled with clay with the density close to that of the Horn of Africa soil in order to enrich the naked mole rats' environment.

Naked mole rats almost immediately began to dig tunnels in the soil, which was considered a normal animals' response. However, half a year later we started noticing exterior changes in some animals. These began to lose weight, their facial features became sharper, which resembled the state of other animals suffering from cachexia [5]. We believed that the reason was loss of the animal's subcutaneous fat. Furthermore, it should be noted that these animals were the first to approach to food, which they did not transfer to the nest, instead of what should have been done if these were workers, but eat it immediately, which suggested their increased need for food. It was decided to remove the compartment with clay and monitor the animals' body mass index throughout three years, with subsequent assessment of microRNAs in the organs that could be damaged: heart, kidney, liver, skeletal muscle. Moreover, to determine the cause of such abnormal animals' condition, histological specimens of the studied tissues and organs were prepared.

It was decided to study microRNA, because the naked mole rats' transcriptome is currently poorly understood, and microRNA is an evolutionarily highly conserved structure involved in regulation of expression of similar genes in different species [6]. The study aimed to determine the cause of such animals' condition.

METHODS

Maintenance and care of animals

The colony of naked mole rats (n = 11) obtained from the Leibniz Institute for Zoo and Wildlife Research (IZW) (Berlin, Germany) was reproduced in the Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, to 54 animals before the study. Each animal had an individual RFID chip implanted subcutaneously for identification in the colony. The colony was kept in cylindrical plastic containers connected to each other with plastic tubes at a temperature of 27 ± 1 °C, humidity 50 ± 10%, and the 12 : 12 h light/dark cycle (10:00–22:00 — light). The diet consisted of apples, sweet potatoes, carrots, cereals provided every day. The animals did not need water, since, due to their physiology, these can get water from solid foods only. To enrich the naked mole rats' environment, a rectangular container with high-density clay mimicking the soil typical for the animals' natural habitat was installed in the colony. The container was removed immediately after the emergence of the signs of cachexia in 9 animals (3 females and 6 males aged 2–6 years) out of 54. To control the condition of these animals, their body mass index was monitored every 4–5 months and compared with that of the control animals of the same age (n = 9; 4 females and 5 males). The animals were euthanized by decapitation after being anesthesized using inhalatory 5% isoflurane (Laboratorios Karizoo. S.A., Spain) with the flow rate of 0.4 L/min in the R500 unit (RWD, China).

Experimental groups of animals for histological assessment and microRNA sequencing

Two groups of naked mole rats were selected for the study: healthy animals (n = 3, one female and two males) with body temperature of 30 °C, body mass index of 0.33, and animals having an asthenic type (n = 3, two females and one male) with body temperature of 27 °C, body mass index of 0.25.

Animals' body mass index calculation

Body mass index (BMI, g/cm^2) was calculated using the following formula: BMI = animal's body weight, g / (animal's body length, cm)^2.

Histological assessment

The animals' liver specimens were fixed with the 10% formalin in 0.1M phosphate buffer (pH = 7.4); dehydrated in five portions of isopropyl alcohol (BioVitrum, Russia), 2 h per portion; soaked in two portions of the Histomix paraffin medium (BioVitrum, Russia), 2 h per portion, and embedded in paraffin blocks. The 3 μm slices were cut using the rotary microtome (Leika, Germany) and stained with Carazzi's hematoxylin and eosin in accordance with the routine protocol. The slides were examined using the AxioScope A1 microscope (Karl Zeiss, Germany). The MRc.5 digital camera (Karl Zeiss, Germany) was used for imaging.

MicroRNA extraction and sequencing

After euthanasia, liver, kidney, heart, skeletal muscle specimens were collected from each animal. RNA fractions containing microRNA were extracted from the tissue specimens using the miRNEasy kits (Qiagen, USA). The samples obtained were later used to create cDNA libraries and for further sequencing. The extracted fraction quality was assessed by microelectrophoresis on Bioanalyzer chips (Agilent, USA). Samples with the RNA integrity number (RIN) of at least 8 were selected for sequencing.

Sequencing involved the use of the NextSeq platform (Illumina, USA) and reagents and disposables from the NextSeq 500/550 High Output v2 kit (Illumina, USA). The cDNA libraries were prepared from the extracted RNA samples using the NEBnext kits (NEB, USA) in accordance with the methods recommended by the manufacturer. Qualitative and quantitative analysis of the libraries was conducted by Bioanalyzer microelectrophoresis (Agilent, USA) and fluorometry in Qubit

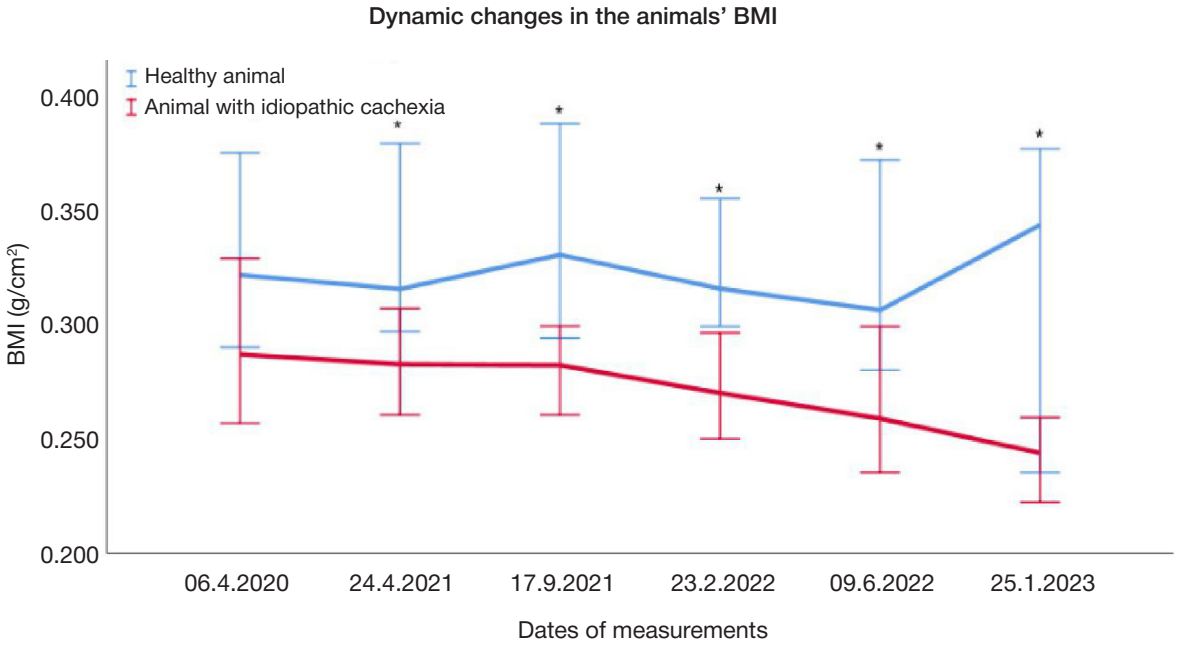

Fig. 1. Curve of the dynamic changes in the animals' BMI. * — $p < 0.05$

(ThermoFisher, USA). The sequencing quality was estimated using the BaseSpace service (Illumina, USA) based on the following parameters: cluster density, signal intensities in detection channels, percentage of clusters passing filter based on the aligned reads output. All the parameters were within the permissible range.

Bioinformatics analysis of microRNA sequencing data

The nucleotide sequences (reads) obtained by sequencing were subjected to mandatory quality control involving the use of the fastqc software tool; high-quality reads were selected for further testing (>30). Adapters were removed using the cutadapt software tool. After adapter removal, only sequences of 18–31 nucleotides corresponding to small RNAs were selected for further assessment.

The search for microRNA was performed using the mirDeep2 algorithm based on the naked mole rat genome and using information about the related genome (Mus musculus). Then random and/or non-specific sequences were delected from the general list of sequences. Only sequences found in more than 60% of samples of the subgroup (experimental or control group for appropriate tissue or organ) that had passed all quality filters were selected for further assessment.

MicroRNA annotation and analysis

The search for human orthologs was performed for all microRNAs that had passed all filters using the blastn software tool; the human microRNA Mirbase v.22 database (https://mirbase.org/) was used as a database for comparison.

The microRNA-target interactions database Mirtarbase v 9.0 (https://mirtarbase.cuhk.edu.cn/~miRTarBase) and miRDB (https://mirdb.org/mirdb) were used to search for possible microRNA targets. Only interactions confirmed by the so-called "strong" evidence (by quantitative PCR, blotting or the use of reporter gene) were selected in Mirtarbase. The microRNA targets having the target score above 80 were selected in the miRDB database.

Enrichment based on various databases, such as GO, KEGG, Reactome, Wikipathways, using the STRING platform (https://string-db.org/) was performed for the microRNA–target pairs selected by this method in order to identify the most involved metabolic pathways and processes.

Statistical data processing

The data were processed in IBM® SPSS® 24 (IBM, USA) using the Mann–Whitney *U* test. The differences were considered significant at $p < 0.05$.

RESULTS

Animals' exterior

In the end of the study the animals with signs of cachexia had a significantly decreased BMI (by 15%) compared to the control animals from the same colony (Fig. 1). Exterior of the animals suffering from cachexia was dramatically different from that of the control animals. These animals had sunken flanks, sharp facial features (Fig. 2).

Histological assessment of the liver

After retrieval of the liver following euthanasia of the animals suffering from cachexia, the liver was deep brown, in contrast to that of the control animals (Fig. 3). Histological assessment of the liver revealed a complex of unique alterations not previously reported for mole rats. All the animals with spontaneous idiopathic cachexia showed accumulation of the large amount of light brown pigment, lipofuscin, in hepatocytes. In some animals, this pigment was more or less evenly distributed across hepatocytes of the liver lobule. In other animals, pigment deposition mainly in pericentral zones with the development of the clearly visible fatty degeneration of hepatocytes was observed. Furthermore, hepatocellular hypertrophy with dramatic enlargement of the cells and nuclei and the emergence of numerous eosinophilic granules (mitochondria) in the cytoplasm was found in periportal areas. In certain animals, the extramedullary hematopoiesis and erythrophagocytosis by hepatocytes phenomena were observed. In healthy animals, there were no lipofuscin or dystrophic changes in hepatocytes (Fig. 4).

Fig. 2. A. Exterior of the healthy animal. **B**. Exterior of the animal with idiopathic cachexia

Fig. 3. View of the liver of the animal with idiopathic cachexia

As is well known, lipofuscin is produced from the remnants of the membranes of intracellular organelles after their degradation in autophagosomes. That is why lipofuscin deposition in hepatocytes is a morphological sign of autophagy intensification and liver cell ageing.

Annotation of naked mole rat microRNA

After filtration of the sequences obtained by sequencing based on the length and quality, microRNA sequences from the naked mole rat liver, kidney, heart, and skeletal muscle were obtained. The overall pool of all the sequences identified was annotated similarly to human orthologs in Mirbase. The naked mole rat microRNA base was created for the first time (Table 1). A total of 162 sequences were reported 90–100% identical to human analogues. Moreover, no human analogues were identified for 22 naked mole rat small RNA sequences. Therefore, it is reasonable to suppose that additional specific regulation of gene expression is typical for naked mole rats. Its adaptive value can be explored in the future by studying the range of microRNAs of the species genetically and environmentally close to naked mole rats (Mus musculus, Cavia porcellus, Ellobius talpinus, Cryptomys damarensis).

Potential targets were analyzed and enrichment based on GO, KEGG, Reactome, Wikipathways was performed for microRNAs identified in the liver, kidney, heart, and skeletal muscle (Table 2–5). The analysis of the signaling pathways and processes related to the microRNAs identified and their target genes has shown that there are numerous multidirectional ones determining a large number of cellular and even extracellular functions that are involved in normal physiology and spontaneous idiopathic cachexia of naked mole rats. In particular, such distant processes are dealt with, as anatomical structure development and double-stranded DNA binding.

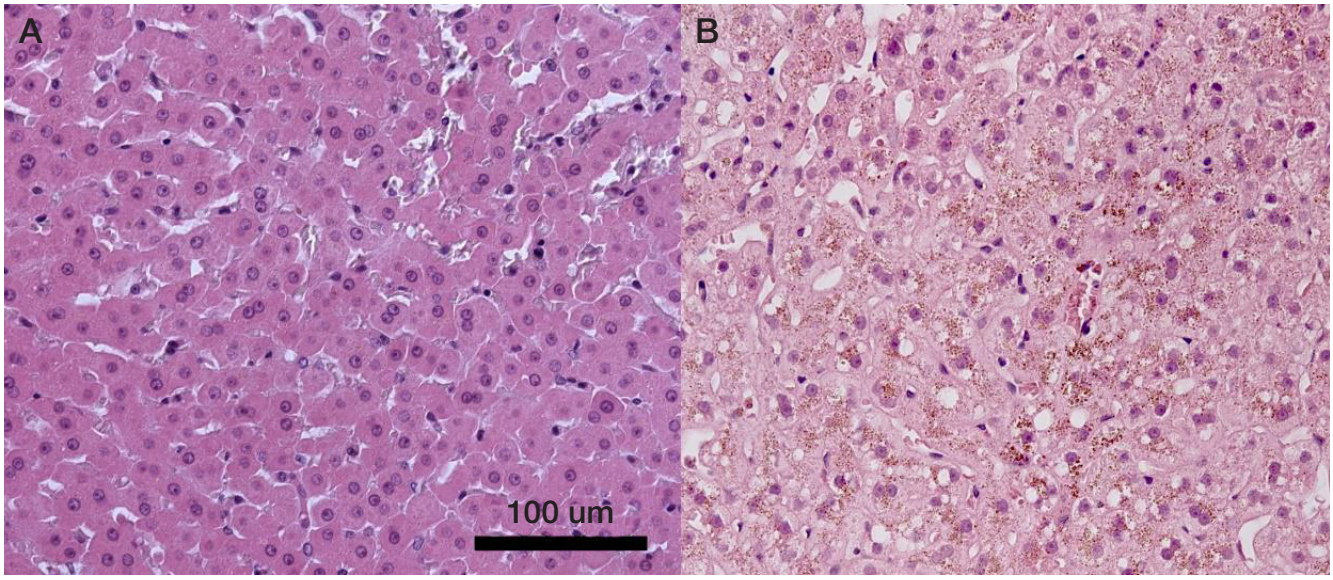

Fig. 4. A. Liver of the healthy animal. **B**. Liver of the animal with idiopathic cachexia. Brown inclusions in the cytoplasm are the lipofuscin deposits. H&E stain, 400x.

Especially noteworthy is the presence of microRNAs responsible for regulation of expression of the genes suppressing apoptosis, but inducing cell division in the naked mole rat heart.

DISCUSSION

The exterior and altered BMI of the animals suffering from cachexia suggest thet these animals failed to gain weight for some unknown reason, but demonstrated the same eating behavior, as other members of the colony, and were even more aggressive with food, since being workers the animals did not carry food to the nesting compartment, but eat it immediately in the feeding compartment.

Perhaps this is because these animals show much higher energy expenditure at rest, than the control animals, for example, as the hyperthyroid induced rats [7]. This is consistent with the data derived from the microRNA sequencing results. The animals suffering from cachexia have the increased share of microRNAs responsible for elevated cell division activity and the decrease in activity of programmed cell death and apoptosis in the heart, which suggests increased cell proliferation in the myocardium followed by its hypertrophy and is also associated with induced hyperthyroidism in mice [8]. Such hypertrophy is essential for the cardiac output increase; when the heart rate is increased, this leads to acceleration of metabolism, which was likely to be demonstrated by the animals unable to gain weight while maintaining the same diet. Such alterations in the heart can result in its failure observed in humans also suffering from hyperthyroidism, which will result in lower life expectancy of the animals suffering from cachexia compared to healthy animals [9].

The reported cachexic condition of the animals was also reflected by the animals' liver. The liver itself was bright brown, almost black. This can be associated with large deposits of lipofuscin in the liver tissue demonstrated by histological assessment. Perhaps, intense mitochondrial remodeling involving the autophagy processes took place in the liver tissue, which resulted from intense oxidative stress [10], since these animals are exposed to low oxygen levels (8–15%) in their natural habitat [11], while in laboratory settings these are kept at 21%, i.e. in hyperoxic environment. In the animals suffering from cachexia, an aggravating factor is increased metabolic rate at rest against the background of cardiovascular system hyperactivation, which entails active functioning of the respiratory system, thereby further loading the cachexic naked mole rat's body with excess blood oxygen levels capable of causing the reported manifestations in the liver [12] and heart.

CONCLUSIONS

Considering the facts, it can be assumed that such a condition of naked mole rats was caused by increased physical exertion in hyperoxic state, which could result in liver damage due to chronic oxidative stress resulting in reduced life expectancy and the development of the disorder in such animals. Such an effect demonstrates possible naked mole rats' vulnerability to increased atmospheric oxygen, which confirms that these are physiologically predisposed to living precisely at low oxygen levels. However, the cause of the animals' metabolic rate increase at rest even after removal of the provoking factor is poorly understood. Maybe this is associated with irreversible effects of the long-term oxidative stress, disturbing regulation of cells and integrative systems, on the body. To find out, it is necessary to conduct further studies of these animals, specifically to carry out transcriptome analysis and assess the expression of the differentially expressed microRNA targets.

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