The effect of exercise on intestinal smooth muscles

Abstract of PhD Thesis

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1. Introduction

Healthy eating and regular exercise are essential for a balanced life. The complexity and benefits of physical activity cannot be overstated, as it positively impacts all areas of our bodily functions. The rapid scientific and technological advancements have fundamentally changed modern life. In the past, simple daily tasks required exhausting, lengthy work, but today machines perform these tasks for us, "saving" us many calories worth of energy. Consider transportation: long walks were once necessary to reach destinations, whereas today we simply get into a vehicle and arrive at our destination in no time. Similarly, the delivery of letters and messages used to require significant effort. Today's generation does not know the experience of sending postcards, as everything can be done instantly from their phones. Reflecting on our daily tasks, it is clear that almost everything can be managed with the push of a button while sitting in a chair. This societal development's negative effects are further exacerbated by the quantity and quality of easily accessible food. Daily eating is no longer a survival risk factor but rather a consumer society's substitute activity, aimed not at fulfilling needs but at obtaining pleasure hormones. Summarizing the decreased energy requirements of the average person and the associated calorie-dense foods, it is not surprising that many "lifestyle diseases" are prevalent, such as obesity, diabetes, cardiovascular, and neurological diseases. The rapid and significant development enabling our convenience has not been matched by human genetics. Our bodies, though slowly, have adapted incredibly well during the caveman era, ensuring survival. At that time, fitness, daily migrations, and the efficient breakdown or storage of nutrients were key to survival. The gastrointestinal tract, including the collection and quality of bacteria called the microbiome, also adapted to this. Initially, at birth, we do not have a gut flora; its development and quality depend on numerous factors. Our age, diet, lifestyle, habits, and the presence of physical activity all influence it. Research has shown the importance of children consuming natural breast milk directly from their mother, not formula through a bottle. This affects the composition of internal bacteria, shaping our immune system's resistance. A 70-kilogram human body contains about 2.5 kg of bacteria, which is ten times the number of human cells in the body. In a healthy body, these microorganisms strive to use nutrients as efficiently as possible. In ancient times, tribes often fought for food, sometimes suddenly having plenty, then having little for weeks. Therefore, the body stored unused food in fat reserves to ensure survival. This explains the interesting dichotomy in today's world, where our genetic coding does not always align with our goals. Individuals with an optimal composition and function of their microbiome will store unused energy as efficiently as possible. Conversely, an

individual with a less efficient internal microorganism composition will be less capable. Our body's primary function is survival, for which it is coded. Today, however, this mechanism is disadvantageous as we no longer live on the savannah, and food acquisition is easy, while our sedentary lifestyle lowers our energy needs. Numerous studies support the systematic effects of exercise in both healthy and diseased individuals. Regular exercise not only increases the body's endurance but significantly contributes to the immune system's resilience, which often begins with the proper functioning of the gastrointestinal tract. Pharmaceutical advertisements and marketing emphasize the importance of maintaining internal gut flora with specially selected bacterial strains like bifidus essensis or bifidus actiregularis. While we have long had knowledge about the human microbiome and the bacteria inhabiting the human digestive tract, their thorough examination has only come to the forefront in the past decade with the rise of molecular technologies. The Human Microbiome Project, launched in 2007 with support from the NIH (National Institutes of Health), aimed to learn more about the human microbiome and determine whether it could influence health and better understand the causes of disease.

The novelty of my dissertation lies in the fact that although various GI segment activity changes have been measured in response to stress, medication, or dietary parameter modifications, the impact of exercise on bowel function and its dynamics is a less explored area. Examining the effects of physical activity on motility, microbiome, cognitive functions, and other physiological parameters in such a complex manner is, to the best of our knowledge, an understudied area. One of the main tasks of my dissertation is to better understand and study these complex processes and their interdependent interactions.

2. Objectives

The main aim of the studies outlined in my doctoral dissertation is to explore the significant correlations along the brain-exercise-gut-microbiome axis. Our hypothesis suggests that the adaptive response induced by physical activity may affect not only individual parts of the axis separately (which many studies have already examined) but also collectively, interacting mutually in a bidirectional relationship. To our knowledge, these correlations and their complexity have not yet been studied in such detail. Therefore, in our research, we conducted biochemical, morphological, and physiological examinations on animals to gain a more comprehensive understanding and better grasp these complex processes and their effects on the body and each other.

The studies in my dissertation can be divided into two fundamental parts. The first phase of our research involved human subjects who underwent a chronic occlusion training method, and we examined the quantitative changes of miRNAs found in skeletal muscle as a result of this. Based on these research results, we formulated the objectives for the second, more prominent part of my dissertation. In this phase, we investigated the long-term effects of exercise on an animal model and its complex composition, with a particular focus on the intestines.
Our hypotheses were formulated as follows:

In the animal model study, we hypothesized that:

1. Voluntary exercise may induce changes in gut motility.
2. Additionally, voluntary exercise influences the microbiome, altering its composition.
3. We hypothesize a correlation between the microbiome and gut motility and their adaptation or modification induced by exercise.
4. We also hypothesize a correlation between the biochemistry of the empty gut and the number and distribution of bacteria found in the microbiome.

In the human study, we hypothesized that:

1. Chronic exercise can influence the quantitative composition of miRNAs, which may exert positive effects even after reaching the intestines.

3. Material and method

3.1. Examination of Voluntary Exercise in Animal Models

3.1.1 Experimental animals

During the research, fourteen middle-aged (11 months old, weight: (591.58 ±60.9g)) male Sprague-Dawley rats (Charles-River Laboratory, Budapest, Hungary) were divided into two groups: a control group (Control=C, n=6) and a voluntarily exercising group (Voluntary Exercise=VE, n=8). The voluntarily exercising group was provided with a running wheel continuously, and the running distances were recorded every day at the same time using a wired bicycle computer (MARWI 5-functional UNION-5N). Before placing the voluntarily exercising group into their running wheels, we measured the maximum oxygen consumption for each animal (VO2max). After 6 weeks of running, the animals underwent the Morris Maze water maze test, and we repeated the VO2max measurement, which was considered a Vita maxima load. Immediately afterward, lactate measurements were performed from the tail vein.

Throughout the study, the animals were housed in pairs in separate cages, subjected to a 12-hour light-dark cycle in their living environment. The animals had ad libitum access to food and water.

3.1.2. Morris Maze Test

For measuring cognitive function and spatial learning, we applied the well-known Morris Maze water maze test. The survey was conducted for 5 consecutive days, immediately after the completion of the exercise program. In a 100 cm diameter and 60 cm height black, circular pool, a platform with a diameter of 6 cm was placed in the middle of the northeast quadrant. The water temperature in the pool was maintained at 22-23 °C, and the filling height was raised 1 cm above the platform until it was covered. Initially, the animals were placed on the resting platform for 30 seconds to familiarize themselves with its exact location in the pool. Subsequently, they were placed at one of the four possible starting points (north, south, west, east). From this point, they had a total of 90 seconds to find the platform again. If this one and a half minutes were not sufficient to locate the resting station, we manually placed them there for an additional 30 seconds. The starting point for the second, third, and fourth attempts was one of the three directions not used previously. The order of the starting points was pseudo-randomly changed for each animal every day. The time needed to find the platform was recorded, the times of the four repetitions were averaged, and changes observed over the five days were analyzed using statistical methods. Reference memory, based on long-term memory, was calculated by comparing the first associations of the test days, while working memory calculations considered the weekly averages of all experiments.

3.1.3. Measurement of Maximum Oxygen Uptake (VO2max)

Familiarization for each animal began with a five-day, ten-minute daily adaptation period on our motorized treadmill (Columbus Inst., Columbus, Ohio). The exact protocol employed aligns with that utilized in our previous studies. In each case, a 5% increment was set on the treadmill, gradually increasing the speed from 8 m/min to 23 m/min. VO2max assessment for each animal was based on three criteria: (i) VO2 remained unchanged with increasing speed, (ii) rats no longer maintained their position on the treadmill, and (iii) respiratory quotient (RQ = VCO2/VO2) > 1.

3.1.4. Maximal Exercise Test and Lactate Measurement

At the end of the exercise program, following the VO2max assessment, the protocol diverged slightly from the first occasion. Animals ran under gradually increasing load until reaching the

point of exhaustion while maintaining a normal position on the treadmill. Subsequently, immediately after the test, blood samples were taken from the animals' tail veins, and lactate levels were measured using the Nova Biomedical Lactate Plus lactate analyzer.

3.1.5. Electromyographic (EMG) Measurement

The most suitable measurement method for our research proved to be the EMG measurement method using TENS electrodes, as employed by Szűcs and colleagues. The primary reason for this choice was the elimination of the need for a subcutaneously insertable bipolar electrode pair. This method, utilizing transcutaneous surface electrodes, allows for the painless, non-invasive measurement of the myoelectric activity of the stomach, small intestine, and colon simultaneously. Moreover, it offers multiple reusabilities. The TENS electrodes (Electrode PE Foam Solidgel, Bio Lead-Lok B Sp. Zo.o, Józefów, Poland) were affixed to the skin without surgery, following a simple depilation, using adhesive tape (Leukoplast 5 cm, BSN medical GmbH, Hamburg, Germany).

To ensure proper conductivity of the electrodes, we used Ten20 EEG conductive gel (Bio-Medical Instruments, USA) on the skin. The standard electrode pairs (2 electrodes) were attached to the right and left sides of the abdominal wall. GIT EMG measurements were conducted after 6 weeks of voluntary exercise with the voluntarily exercising group and control animals simultaneously, under stress-free conditions, between 9:00 and 11:00 AM at room temperature (24 °C). Prior to this, a 2-week familiarization period was applied to each animal to introduce the EMG boxes without creating a new environment, ensuring the calmest possible measurements. Before each measurement, the animals underwent a form of "metabolic cage" by fasting to exclude EMG signals derived from digestion.

We recorded the examined electrophysiological parameters for a minimum of 30 (preferably 90) minutes and analyzed them using an online computer signal amplifier system, namely the 10 S.P.E.L. Advanced ISOSYS data acquisition system (MDE GmbH, Walldorf, Germany). The need for extended recordings was due to the detection of the longest resting signals, for which we later employed a digitally integrated cutting program. The EMG signals were initially amplified using a custom-designed amplifier by MDE Ltd. (Budapest, Hungary). To reduce artifacts, a dual-filter system was used. We pre-filtered all analog signals with a first-order Bessel-type low-pass filter, then converted them into digital signals with a 2 Hz sampling frequency and an 80 dB/decade slope. The pre-filtered myoelectric signals were further filtered using Bessel-type band-pass filters with a frequency range of 0-30 cycles per minute (cpm), with a slope of 140 dB/decade. All filters were digital IIR filters. We analyzed the recorded

signals using Fast Fourier Transformation (FFT). The frequency of electrical activity was characterized in cpm, while the magnitude of activity was described as power spectrum density (PsD). Regarding the interpretation of physiological parameters, if values exceeded one standard deviation, we considered them as outliers, excluding them from the analysis, as described earlier.

3.1.6. Methods Used for Detecting Biochemical Changes

After the completion of the exercise program and subsequent physiological tests, animals were anesthetized intraperitoneally using ketamine (Richter, concentration: 100 mg/ml) /xylazine (Produlab Pharma, concentration: 20 mg/ml) injection at a dose of 0.1 ml/10g body weight. They were then transcardially perfused with heparinized, ice-cold saline solution. Following the opening of the abdomen, approximately 5 mm rings were quickly removed from the descending colon and ~20 mm rings below the ileocecal junction, while larger segments (~30 mm) were collected for transmission electron microscopy (TEM, n=5 groups each). Unaffected mitochondria and whole protein extracts were obtained from the intact colon.

3.1.7. Western Blot Molecular Biology Procedure

The Western blot procedure is a semi-quantitative method suitable for the immunodetection of specific proteins in various tissue samples. In this study, we examined the quantity of different proteins present in the colon.

Samples extracted from the middle part of the colon were homogenized using a Turrax homogenizer (IKA T10 basic ULTRA-TURRAX DISPERSER 50/60Hz, Staufen im Breisgau, Germany) with lysis buffer (137mM NaCl, 1% NP 40, 10% glycerol, 20mM Tris pH 8.0) added on ice. Protease/phosphatase inhibitors [Aprotinin (2μg/ml), Leupeptin (5μg/ml), PMSF (1mM), Na orthovanadate (1mM)] were also added. The mixture was then shaken on ice for 40 minutes, followed by centrifugation at 15,000g, and the supernatant was pipetted off.

Protein measurement was performed using the Bradford (BioRad Protein Assay, Dye Reagent Concentrate) and Lowry protocols. Samples were applied in triplicate, measured at a wavelength of 595 nm using Multi Scan EX (Thermo Labsystem) equipment, and then diluted to a uniform concentration. Sample dilution was performed with 2X Laemmli buffer, followed by heating at 90 °C for 5 minutes. The resulting samples, ready for analysis, were stored at -80 °C.

Throughout processing, samples were always examined in 10% Sodium Dodecyl Sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, which took approximately 1-1.5 hours at a constant voltage of 150 V, using Biorad marker (Biorad 1610374) during pocket filling.

After electrophoresis, a pre-cut PVDF membrane was activated in methanol for 1 minute, then soaked for 5 minutes in 20% methanol transfer buffer with a blotting paper. Subsequently, proteins were transferred from the gel to the membrane using a transfer unit, creating a so-called "sandwich" consisting of the membrane, gel, and blotting paper. Each component of this sandwich was moistened with 20% methanol transfer buffer to prevent drying. The transfer was carried out for 1.5 hours at a constant voltage of 30 V.

Following transfer, the membrane was placed in Tris-buffered saline-Tween 20 (TBST) solution for 1 minute, while the gel was immersed in a gel-staining solution on a shaker for 2 hours (50% methanol, 39.75% H2O, 10% acetic acid, 0.25% Coomassie Brilliant Blue). After 1 minute, the membrane was transferred to TBST with either 0.5-5% milk powder or 5% BSA for 2 hours. This blocking was also performed at 4 °C on a shaker. The blocked membrane was then treated overnight at 4 °C with the appropriately diluted primary antibodies corresponding to the target proteins (see Table 1). The dilution of primary antibodies was always performed according to the instructions of the antibody-producing company. The next morning, the membrane was washed three times for 20 minutes each with TBST to remove non-specific bindings.

The next step involved treating the membrane with secondary antibodies at 4 °C for 2 hours on a shaker. During incubation, peroxidase-conjugated secondary antibodies (Jackson 1:10000) from mouse, goat, and rabbit were used. Subsequently, the membrane was washed again three times for 20 minutes each with TBST at room temperature while shaking. After the washes, the membrane was incubated with horseradish peroxidase for 1 minute, protected from light, also at room temperature. The incubated membrane was then visualized using the AZURE 400 visible fluorescence imaging instrument (version 1.7.6.1202). The bands appearing at the appropriate molecular weights, representing our target proteins, were evaluated using ImageJ software, with relative density always calculated against the "housekeeping" protein.

As a final step, the membrane was stained with a membrane staining solution (0.2% Coomassie Brilliant Blue, 45% methanol, 10% acetic acid, 44.8% H2O). The stained membrane, along with the previously developed images, was also evaluated using the ImageJ program. The housekeeping protein in all our measurements was tubulin.

3.1.8. Preparation of Mitochondrial, Cytosolic, and Nuclear Fractions

We used a protocol employed by Scorrano and colleagues for fractionation, with minor modifications. The entire procedure was carried out at 4 °C. Fresh connective tissue- and fat-free colon tissue was immersed in ice-cold PBS supplemented with 10 mM EDTA and minced

into the smallest possible pieces. The samples were then homogenized using a Turrax homogenizer (IKA T10 basic ULTRA-TURRAX DISPERSER 50/60Hz, Staufen im Breisgau, Germany). Sample exposure was carried out with mild shaking (200 rpm) for 30 minutes using 0.05% trypsin, followed by centrifugation at 1000g for 5 minutes. The pellet was reserved for additional Western blot analysis, including the pellet and the cytosolic supernatant.

After gently removing the sample from the centrifuge, the supernatant was aspirated off, and the remaining mitochondria and cytosolic components were transferred separately to a new Eppendorf tube. From the sediment at the bottom, 100 µl was gently transferred to a new Eppendorf tube with 20 µl NP40 lysis buffer and 66 µl 4XSDSDDT to avoid clumping. This was suspended, shaken gently, and homogenized with a sonicator for 2 minutes at intensity 3-4 until a whitish light-blue color was obtained. Subsequently, it was boiled for 5 minutes at 100 °C and centrifuged again at 14,000 rpm. The supernatant was collected, and the Nuclear fraction was obtained, stored at -70 °C until later use.

We repeated the centrifugation step with the eppendorf containing the reserved mitochondrial and cytosolic fractions after homogenization in IBm1 buffer to obtain high-quality intact mitochondria. This procedure was carried out at 8000 g (14000 rpm) for 10 minutes at 4 °C. From the supernatant representing the cytosolic fraction, we carefully transferred 100 µl to a clean eppendorf containing 20 µl of NP40 lysis buffer and 66 µl of 4XSDSDDT. Subsequently, we suspended, mixed, and homogenized the samples using a sonicator for 2 minutes at 3-4 intensity, followed by boiling at 100 °C for 5 minutes. Afterward, the samples were stored at -70 °C for future use. The mitochondrial pellet was suspended in the minimal amount of IBm2 buffer (10 mM Tris-HCl, 3 mM Tris-EGTA, and 0.25 M sucrose, pH 7.4). Protein concentration was determined using the Bradford assay, as mentioned earlier.

3.1.9. Reactive Oxygen Species (ROS) Production Assessment

Mitochondria (0.3 mg/ml) were incubated in our experimental buffer (10 mM Tris/HCl, 5 mM MgCl2, 2 mM KH2PO4, 20 mM EGTA/Tris, 250 mM sucrose, pH 7.4) supplemented with 1 µM Amplex Red (excitation: 560 nm; emission: 584 nm) and horseradish peroxidase (10 U) to evaluate ROS production, monitoring fluorescence induced by H2O2. This was performed following a previous study by Votyakova et al. with slight modifications. After measuring basal ROS production, 10 mM succinate (Suc) and/or 1 µM rotenone were added. For succinate as a substrate, ROS production at complex I level would increase along with the reverse electron flow-related ROS production. Rotenone was used to inhibit this effect. The calibration of hydrogen peroxide (H2O2) production was achieved by adding a known quantity of H2O2.

Fluorometric assessments were conducted at 30 °C using the Fluorskan Ascent FL fluorimeter with 96-well plates, and each point was measured in triplicates.

3.1.10. 16S rRNA Gene Amplicon Sequencing

The analysis of microbiome characteristics was performed by analyzing fecal samples from animals, collaborating with SeqOmics Ltd.

The methodology followed our previous human study during COVID-19 infection, where we analyzed the microbiome of active and inactive individuals. Fecal samples were immediately frozen in liquid nitrogen after collection and stored at -80 °C for long-term preservation. Total DNA isolation was carried out for each sample using the DNeasy PowerSoil kit according to the manufacturer's protocol (cat. no. 12888-100; Qiagen GmbH, Hilden, Germany). The V3-V4 regions of the 16S rRNA gene were amplified by Polymerase Chain Reaction (PCR) using the primers: 16S Amplicon PCR Forward Primer = 5'-TCGTCGGCAGCGTCAGTCAGATGTGTGTATAAGAGAGACAGCCTACGGGNGGGCWGCAG, and 16S Amplicon PCR Reverse Primer = 5'-GTCTCTCGTGGGCTCGGAGATGTGTGTGTATAAGAGAGACAGGACTACHVGGGTATCTAATCC. DNA library preparation for Illumina MiSeq sequencing was conducted using the Nextera XT DNA Library Prep Kit (Illumina Inc., CA, USA) according to the manufacturer's protocol. Sequencing was performed on the Illumina MiSeq 2 × 300 bp platform using the MiSeq v3 Reagent Kit (Illumina Inc., CA, USA), with sequencing reads limited to 10,000 per sample before further analysis.

3.1.11. Transmission Electron Microscopy (TEM) Examination of Caveolae

The preparation of sections was assisted by the staff of the Department of Anatomy, Cell, and Developmental Biology at Eötvös Loránd University. For TEM analysis, the samples were fixed in modified Carnovsky fixative (3.2% PFA, 0.2% glutaraldehyde, 1% sucrose, 40 mM CaCl2 0.1M cacodylate buffer) for 24 hours at 4 °C. Subsequently, the samples were cut into two equal parts in the midsagittal plane before being fixed for an additional 12 hours at 4 °C in 5% glutaraldehyde / 0.1 M cacodylate buffer, and then dehydrated with gradually added ethanol until saturation with propylene oxide. The integrity and orientation of the tissues were checked on 0.8-1 µm semi-thin sections before embedding in low viscosity Spurr epoxy resin. Two mini-blocks (1 mm2) were cut from each segment, where the "E" mini-block contained the entire mucosa (epithelium, lamina propria, muscularis mucosa), and the "M" mini-block contained the submucosa, muscularis propria, and serosa layers. The "E" mini-blocks (2/animal, 10/animal group) were used for morphometric analysis of epithelial cells, while the "M" mini-

blocks were used to quantify the number of caveolae in the innermost smooth muscle cells (SMC) of the circular muscle layer (CML). For each mini-block, 6 ultra-thin sections (50-60 nm) were cut using the Reichert OMU3 ultramicrotome and collected on copper grids coated with Formvar (Agar Sci., Essex, UK). The sections were stained with uranyl acetate and Reynolds lead citrate for contrast. Morphometric analysis of caveolae number and plasma membrane length (C – 386467.82±160708.19 nm, VE – 414775.53±226673.91 nm) were performed on microphotographs taken at the same magnification (x 30,000), covering the entire length of the selected SMC. Measurements were conducted using the iTEM software measurement function.

3.1.12. Morphological Measurements

The morphology of mitochondria and vesicles was analyzed using the APEER Online Machine Learning Platform, in conjunction with Zeiss and Fiji programs. Distinguishable mitochondria and vesicles were tracked using a predefined deep learning method based on a previous survey, applied to the colon samples of adult rats (5 animals/group, 3-4 independent images at 20,000× magnification). The Apeer program-driven deep learning created three distinct groups among the classes of mitochondria based on their morphological appearance: "Group A" consisted of normal mitochondria with a dense matrix and parallel, narrow cristae; in the case of "Group B," we observed disordered mitochondria with a still dense but disordered matrix and some lipid droplets; and "Group C" contained swollen mitochondria with a wide extension or complete absence of cristae and significant loss of internal structure. The identified mitochondria and/or vesicles were re-evaluated. The results obtained were first exported to the Fiji program, which provided the following parameters: relative mitochondrial and vesicular area, mitochondrial size; perimeter; aspect ratio (AR, length-width ratio) as [(major axis)/(minor axis)]; form factor (FF, reflecting the complexity of mitochondria) as [(perimeter^2)/(4π * area)]. For further data analysis, the calculated values were imported into the Prism 6 (GraphPad Software) program. Statistical significance was assessed based on the 95% confidence interval (C.I.) of the average.

3.2. Application of Chronic Occlusion Method in Human Model

3.2.1. Subjects

Twenty-two healthy young males volunteered for our study. Subsequently, participants were randomly divided into two equal groups, an occlusive (O – age: 23.9 ± 1.7 years, weight: 77 ± 8.9 kg, height: 182.3 ± 7.8 cm, load unit: 82.2 ± 18.6 kg) and a control (C – age: 24.1 ± 6.1 years, weight: 81.3 ± 6 kg, height: 184.5 ± 7.2 cm, load unit: 86.7 ± 12.9 kg) group. All participants received detailed information about the purpose of the study and its procedures.

Prior to commencing the research, all underwent a medical examination and completed a medical questionnaire to exclude any cardiovascular, metabolic, neurological, or musculoskeletal disorders. The study was approved by the Ethical Committee of the University of Physical Education (ET-KEB/No8/2017), and conducted following the principles of the Helsinki Declaration. Each participant's maximum strength and endurance were assessed before and after the exercise program. Additionally, muscle biopsies were taken twice for subsequent laboratory analysis.

3.2.2. Measurement of Maximum Strength

One week before the commencement of the occlusive exercise program, each subject underwent a maximum strength assessment using a squat rack on a fixed track to prevent injuries. Squatting was a familiar motion for all subjects, who had previously performed similar strength exercises. The maximum strength assessment was conducted using the three-repetition maximum (3RM) test according to the guidelines of the National Strength and Conditioning Association. The test was preceded by a 10-minute warm-up on a stationary bicycle ergometer. During the test phase, participants performed ten repetitions with resistance equal to half their body weight, followed by a set of 4-6 repetitions with their full body weight. In the final stage, volunteers had four attempts to reach their 3RM. The achieved value was defined as 90-93% of the one-repetition maximum (1RM). Resistance was gradually increased, and between sets, participants rested for at least two but no more than five minutes. A repetition was considered successful if no external assistance was required to move the weight and if the thigh reached a parallel position with the ground.

3.2.3. Exercise Protocol

Three days after the last muscle biopsy and four days before the first exercise session, maximum strength was measured using the 3RM test. After two rest days, an endurance test was conducted, with the load set at 70% of 1RM, determined by full squat strength testing. Both tests were performed before and after the four-week training sessions.

3.2.4. Biopsy Sampling

Three days before the commencement of the exercise program, the first muscle biopsies were taken from the study subjects, and the second biopsies were taken 24 hours after the completion of the exercise program. Biopsy sampling was performed using a semi-automatic needle (EASY-RAM 14Gauge 100 mm (length)) with local anesthesia (20 mg/ml lidocaine hydrochloride; EGIS, Budapest, Hungary) by a trained individual from the right vastus lateralis

muscle. Subsequently, muscle samples were rinsed with PBS pH 7.4 solution and immediately frozen using liquid nitrogen. The samples were stored at -80°C until further miRNA analysis.

3.2.5. RNA Extraction for Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) for MicroRNA Transcript Analysis

Quantitative real-time polymerase chain reaction (qRT-PCR) was employed for miRNA analysis of the samples. RNA extraction from 5-10 mg muscle tissue pieces was performed using Trizol reagents (TRI Reagens®: TR 118, MRC Inc., Cincinnati, OH, USA). RNA purity was assessed based on absorbance measurements at 260 nm and 280 nm wavelengths. Extracted RNA with a ratio greater than 1.8 was used for subsequent analysis. Total RNA (10 ng) was reverse transcribed using the MicroRNA Reverse Transcription Kit (TaqMan™ Advanced miRNA cDNA Synthesis Kit: A28007, Applied Biosystems, Foster City, CA, USA) following the manufacturer's protocol with slight modifications for individual target miRNAs quantity determination. TaqMan MicroRNA Assays for miR-486, miR-499, miR-206, miR-1, miR-133a, and miR-233b were utilized (TaqMan™ Advanced miRNA Assay: A25576, Applied Biosystems). qRT-PCR was performed using the TaqMan Fast Universal PCR Master Mix (Cat#: 4366072, Applied Biosystems) in a 7500 Fast Real-Time PCR System according to the manufacturer's protocol. All samples were run in triplicate, and normalization was performed as described previously, using miR-191 as the endogenous control. Each reaction was run separately, and quantification was performed using the Ct (ΔΔCt) method.

3.3. Statistical Analysis

In the Morris maze test conducted on the animal model, group differences were examined using two-way ANOVA, and group means were compared using Tukey's test. Changes in maximum oxygen consumption in animals were measured using a one-sample T-test. To assess differences between the control and exercise groups (following the vita maxima test, lactate levels, EMG measurements of bowel peristalsis, mitochondrial and free radical measurements in bowel sections, Western blot analysis of proteins, and morphological measurements of bowel sections), two-sample T-tests were applied. Normal distribution was assessed using the Shapiro-Wilk test. If normal distribution was not found, the Kruskal-Wallis test was applied instead of Tukey's test. All bioinformatics analyses related to the microbiome were conducted using the Snaq program, and analysis of 16S microbiome data was performed using the QIIME2 program. The DADA2 algorithm was used for amplicon sequence variant inference, and taxonomic classification was performed using the SILVA 128 reference database, from phylum to genus level. Within-sample species diversity was measured using the Shannon diversity

index. In the human model, normality was assessed using the Shapiro-Wilk test, and all presented variables followed a normal distribution. A one-sample T-test was used to determine statistical differences before and after exercise, and a two-sample T-test was used to identify differences between groups. Pearson correlation was used for correlation analysis, and significance was determined at $p<0.05$.

4. Results

4.1. Results of Voluntary Exercise Animal Model

4.1.1. Morris Maze Test Values

In the Morris Maze water maze, although there was a trend in both reference and working memory, with VE group animals finding the hidden platform in the pool in less time compared to control animals, significant differences were observed only on the second (C – 70.55±27.68 sec, VE – 45.13±36.85 sec, $p < 0.001$) and fourth days (C – 46.86±28.49 sec, VE – 30.75±30.64 sec, $p < 0.001$) for working memory. Working memory was calculated as the average of 4 trials each day spent in the water, while reference memory was calculated as the average of the first trials on each day. Results are presented as the average ± SE for control (C, n=6) and voluntarily exercising (VE, n=8) rats.

4.1.2. Results of Maximum Oxygen Uptake (VO2max)

A significant increase in VO2max level was observed in the VE group (62.95±14.53 ml/kg/min → 75.32±11.56 ml/kg/min, $p < 0.001$), while a significant decrease was noted in the control group (65.77±14.18 ml/kg/min → 60.69±9.09 ml/kg/min, $p < 0.05$).

4.1.3. Vita Maxima Procedure and Lactate Measurement Results

Following the Vita maxima test, the lactate levels obtained after blood sampling showed significantly lower values in the VE group compared to the C group (C - 6±2.8 mmol/l, VE – 3.2±1.4 mmol/l, $p < 0.001$).

4.1.4. Results of Electromyography (EMG) Measurements

The results of smooth muscle EMG measurements were similar between the VE and C groups. However, in every tract measured for the VE group (small intestine, colon, and stomach), the myoelectric signals of smooth muscles were smaller. Nevertheless, no significant correlation was found in these measurements, only a slight trend.

In the case of both control and exercised rats, there were no significant differences in the electric signal activity of the small intestine (C – 1.133±0.51 μV^2, VE – 0.887±0.51 μV^2), colon (C – 2.273±0.73 μV^2, VE – 1.99±0.91 μV^2), and stomach (C – 2.777±1.34 μV^2, VE – 2.493±1.31 μV^2). The evaluation of signals was based on the method developed by Szűcs and colleagues.

4.1.5. Results of Western Blot Molecular Biology Procedure

Western blot analysis of colon samples revealed significantly increased levels of eNOS ($p < 0.05$) and Akt1 ($p < 0.01$) in the VE group, while no significant changes were observed in the SIRT1, SIRT3, NRF1, PGC-1α, NFκB, CS, and NAMPT protein levels.

4.1.6. Results of Reactive Oxygen Species (ROS) Production Examination

Examining the ROS production in freshly isolated mitochondria from the colon, no significant differences were found between the C and VE groups. Thus, voluntary exercise did not result in a significant change in ROS production, neither before nor after succinate administration. Rotenone treatment following succinate addition, however, led to complete inhibition of ROS production in all cases.

4.1.7. Results of 16S rRNA Gene Amplicon Sequencing

During microbiome analysis, very similar data were observed for the Shannon diversity index of bacterial cultures. The exercise increased the relative abundance of Actinobacteria, one of the four main species of the gut microbiota. At the Family level, changes in abundance revealed an increase in Bifidobacteria and Ruminococcaceae, presumably due to increased SCFA production. Although NF-κB did not change, the abundance of Acetatifactor decreased in the VE group compared to the C animals.

4.1.8. Examination of Relationships

Analyzing the correlations of the measured data revealed that lactate levels negatively correlated with Akt and eNOS levels ($r=-0.646$ and $r=-0.511$, respectively) and positively correlated with NRF1 levels ($r=0.742$). Spatial memory showed a negative correlation with VO2max levels ($r=-0.457$). The quantitative occurrence of Bifidobacteriales and Bifidobacteriaceae in the microbiome demonstrated a correlation with VO2max levels ($r=0.436$).

4.1.9. Morphological Measurement Results

Based on electron microscopic data, it was observed from the morphological examination of mitochondria that although VE increased the quantity of mitochondria, it also caused the elongation of normal mitochondria. Controlled by the Apeer program, deep learning created three different groups among the classes of mitochondria based on their morphological appearance: In the (a) group, normal mitochondria with a dense matrix and parallel narrow cristae were present; the (b) group consisted of disordered mitochondria, still with a dense matrix but now with disordered cristae and some lipid droplets; the (c) group included swollen mitochondria, showing a wide or complete lack of cristae and significant internal structure loss. The Apeer program also identified the vesicles.

The quantification was based on the glass-shaped membrane invaginations measured with a microscope (C – 16.24±1.24 µm, VE – 24.14±2.67 µm), which seemed to increase in number in the GIT wall due to the effect of VE.

During quantification, only those flask-shaped membrane formations were considered that exhibited visible openings on the cell surface or had the membrane covered with an opening. Membrane formations that were elongated or did not show contact with the plasma membrane in the section plane were not taken into account.

4.2. Results of Chronic Occlusion Method in Human Model

In the human study, both miRNS-1 (C - 7.45±1.78 → 7.31±2.29, O - 7.71±1.00 → 5.62±2.37, $p<0.05$) and miRNS-133a (C - 11.11±2.56 → 10.76±3.97, O - 12.06±2.1 → 9.07±4.40, $p<0.05$) levels significantly decreased in the muscle samples of individuals undergoing occlusion compared to the control group. Remarkably, we also found a significant positive correlation between the relative expression levels of these two miRNAs, which may indicate a common physiological regulation.

5. Conclusions

5.1. Voluntary Exercise May Induce Changes in Bowel Motility

Results from EMG measurements of the rat's smooth muscle during exercise showed similar outcomes between the VE and C groups when observing the myoelectric activity of the small intestine, stomach, or even the colon. Although a certain tendency is noticeable, with the myoelectric signals of control animals being higher compared to the VE group, significant differences could not be established between the two groups, so we DO NOT ACCEPT this claim.

Based on these data, we conclude that in response to such mild-intensity, predominantly aerobic exercise, the organism does not necessarily attempt to adapt to stimuli by modifying motility. It is conceivable that processes targeting biochemical and/or structural changes may prove to be a more effective strategy for the organism, possibly achieving adaptation by modifying the microbiome composition.

It is important to note, however, that motion artifacts posed the greatest limiting factor in recording the EMG signals of rats performing voluntary exercise. For this reason, many data points had to be excluded from our measurements. In future research, using a larger sample size and/or more familiarization might help mitigate the occurrence of artifacts, preserving more data for later analysis. Thus, the currently observed trend could potentially represent a significant difference in the future.

5.2. Voluntary Exercise Influences the Microbiome, Capable of Altering its Composition

Considering the Shannon diversity index of bacterial culture during 16S rRNA gene amplicon sequencing, very similar data were observed for the C and VE groups. Nevertheless, we ACCEPT the statement since voluntary exercise increased the relative abundance of Actinobacteria, one of the four main phyla of gut microbiota. At the Family level, the relative abundance of Bifidobacteria and Ruminococcaceae increased compared to the C group, presumably due to increased short-chain fatty acid production. At the Genus level, the abundance of Acetatifactor decreased in the VE group compared to the C group.

These results confirm the positive effects of exercise on the microbiome, which play a crucial predictive role against diseases and have implications for overall fitness.

5.3. Relationship, Correlation Between the Microbiome and Bowel Motility, their Exercise-Induced Adaptation, and Modification

No direct correlation or relationship could be established between the data on gut microbiome and GIT myoelectric activity. Thus, we DO NOT ACCEPT this claim.

However, since the gut-brain-exercise-microbiome axis forms a very complex, highly intricate system, with each component capable of regulating the other through numerous pathways, it is possible that they can indirectly influence each other. Therefore, we do not entirely rule out the possibility of retesting this hypothesis in future research within a different experimental design or with a larger sample size.

5.4. Connection Regarding the Data on Bowel Biochemistry and the Number and Distribution of Bacteria in the Microbiome

During Western blot analysis of colon samples, significantly higher levels were observed for the eNOS and Akt1 proteins, while no significant changes were detected in the levels of SIRT1, SIRT3, NRF1, PGC-1α, NFkB, CS, and NAMPT proteins in the voluntarily exercising group compared to the control.

Examining the ROS production of freshly isolated mitochondria from the colon, no significant differences were found between the C and VE groups. Therefore, the method of voluntary exercise did not result in a significant change in ROS production, either before or after succinate administration. Succinate administration followed by rotenone treatment consistently led to complete inhibition of ROS production in all cases.

Analyzing the correlations of the measured data, it can be concluded that lactate levels negatively correlated with Akt and eNOS levels, and positively correlated with NRF1 levels. Spatial memory also negatively correlated with VO2max levels. The quantitative occurrence of Bifidobacteriales and Bifidobacteriaceae in the microbiome showed a correlation with VO2max levels.

From the aspect of this hypothesis, taking into account the single, crucial, and outstanding correlation we found, which is the correlation between the Akt1 protein content and the quantity of Bifidobacteria in the stool, we ACCEPT this claim. The Akt protein plays several physiologically important roles, including its known association with peripheral glucose uptake and its impact on insulin sensitivity. Some studies have linked its signaling role to depression originating in the brain.

In conclusion, we ACCEPT the claim that there is a connection between the data on bowel biochemistry and the number and distribution of bacteria in the microbiome.

5.5. Chronic exercise can influence the quantitative components of miRNAs, which, upon reaching the gastrointestinal tract, may exert positive effects.

Significant decreases were observed in the levels of miRNS-1 and miRNS-133a in the muscle samples of the occlusion exercise group compared to the control group, thus we ACCEPT this statement.

It is important to emphasize that these non-coding RNAs, through highly complex processes, can participate in numerous regulations, both in the muscle and in other areas of the body reached by the bloodstream. To further map out their multifaceted, interacting mechanisms, it would be essential to re-examine the results from different perspectives.

6. List of own publications

Announcements related to the dissertation

Peter Bakonyi, Attila Kolonics, Dora Aczel, Lei Zhou, Soroosh Mozaffaritabar, Kinga Molnar, Lajos Laszlo, Balazs Kutasi, Kumpei Tanisawa, Jonguk Park, Yaodong Gu, Ricardo A. Pinho, Zsolt Radak. 2023. „Voluntary exercise does not increase gastrointestinal motility but increases spatial memory, intestinal eNOS, Akt levels, and Bifidobacteria abundance in the microbiome". Frontiers in Physiology Volume 14 – 2023. https://doi.org/10.3389/fphys.2023.1173636.

Torma, Ferenc, Peter Bakonyi, Zsolt Regdon, Zoltan Gombos, Matyas Jokai, Gergely Babszki, Marcell Fridvalszki, és mtsai. 2021. „Blood Flow Restriction during the Resting Periods of High-Intensity Resistance Training Does Not Alter Performance but Decreases MIR-1 and MIR-133A Levels in Human Skeletal Muscle". Sports Medicine and Health Science 3 (1): 40–45. https://doi.org/10.1016/j.smhs.2021.02.002.

Announcements not related to the dissertation

Torma Ferenc, Gombos Zoltán, Bakonyi Péter, Radák Zsolt. „Az edzés pihenő idejében alkalmazott okklúzió hatása négyhetes guggoló edzést végző egyének átlagsebesség mutatóira. Testnevelés, sport, tudomány – Physical, Education, Sport, Science - Original Research Paper (96): 3-4. http://doi.org/10.21846/TST.2018.3-4.5.

Gergely Babszky, Ferenc Torma, Dora Aczel, Peter Bakonyi, Zoltan Gombos, Janos Feher, Dora Szabo, Balázs Ligeti, Sándor Pongor, Laszlo Balogh, Anikó Pósa, Zsolt Radak. 2021. „COVID-19 Infection Alters the Microbiome: Elite Athletes and Sedentary Patients Have Similar Bacterial Flora". Genes 2021, 12(10), 1577; https://doi.org/10.3390/genes12101577

Gombos, Zoltan, Erika Koltai, Ferenc Torma, Peter Bakonyi, Attila Kolonics, Dora Aczel, Tamas Ditroi, Peter Nagy, Takuji Kawamura, és Zsolt Radak. 2021. „Hypertrophy of Rat Skeletal Muscle Is Associated with Increased SIRT1/Akt/MTOR/S6 and Suppressed Sestrin2/SIRT3/FOXO1 Levels". International Journal of Molecular Sciences 22 (14): 7588. https://doi.org/10.3390/ijms22147588.

Dora Aczel, Bernadett Gyorgy, Peter Bakonyi, RehAn BukhAri, Ricardo Pinho, Istvan Boldogh, Gu Yaodong és Zsolt Radak. „The Systemic Effects of Exercise on the Systemic Effects of Alzheimer's Disease". Antioxidants 2022, 11(5), 1028; https://doi.org/10.3390/antiox11051028.

Matyas Jokai, Ferenc Torma, Kristen M. McGreevy, Erika Koltai, Zoltan Bori, Gergely Babszki, Peter Bakonyi, Zoltan Gombos, Bernadett Gyorgy, Dora Aczel, Laszlo Toth, Peter Osvath, Marcell Fridvalszky, Timea Teglas, Balazs Ligeti, Regina Kalcsevszki, Aniko Posa, Sylwester Kujach, Robert Olek, Takuji Kawamura, Yasuhiro Seki, Katsuhiko Suzuki, Kumpei Tanisawa, Sataro Goto, Istvan Boldogh, Xueqing Ba, Dora Szabo, Kelvin J. A. Davies, Steve Horvath, Zsolt Radak. „DNA methylation clock DNAmFitAge shows regular exercise is associated with slower aging and systemic adaptation". Geroscience 2023 May 20. https://doi.org/10.1101/2022.07.22.22277842.