Time frame of the extracellular vesicles' release after high intensity exercise

Az extracelluláris vezikulák felszabadulásának időbeli vizsgálata magas intenzitású edzés után

Bernadett Gyorgy[1], Ferenc Torma[1], Gergely Babszky[1], Matyas Jokai[1], Zoltan Gombos[1], Peter Bakonyi[1], Edit I Buzas[2], Krisztina Paloczi[2], Tamas Szabo[3], Zsolt Radak[1], Erika Koltai[1]

[1]Research Center for Molecular Exercise Science, Institute for Sports and Health Sciences, University of Physical Education, Budapest Hungary
[2]Department of Genetics, Cell and Immunobiology, Faculty of Medicine, Semmelweis University, Budapest, Hungary
[3]Research Center for Sport Physiology, University of Physical Education, Budapest Hungary

E-mail: gyorgy.bernadett.tf@gmail.com

Abstract

Exercise related adaptation is systemic, however the adaptation paths are not completely discovered. Extracellular vesicles (EVs) could be involved in the communication between organs. We assumed that a single bout of anaerobic exercise increases the concentration of vesicles in the blood. The aim of this study was to investigate the effect of high intensity exercise on extracellular vesicles, which were isolated and analyzed from human venous blood. This method can help us understand more about the time frame and size distribution of the vesicle release into the blood circulation. Young, healthy, male volunteers (n=12) participated in our pilot study. Blood samples were collected before, right after, five and 24 hours later after the high intensity exercise and used for the separation of EVs. After differential centrifugation and size-exclusion chromatography, two different methods were used to count the amount of medium and small size EVs. Results revealed huge individual differences. It seems that 5 hrs after the exercise bout is an appropriate time point to collect EVs.

Keywords: extracellular vesicles, high intensity exercise

Összefoglaló

A rendszeres testmozgás szisztémás hatásai jól ismertek, azonban az adaptációs útvonalak még nem teljesen feltárt terület. Előzetes kutatások alapján az extracelluláris vezikulák (EVs) részt vehetnek a szervek közötti kommunikációban. Úgy gondoljuk, hogy akut intenzív fizikai terhelés hatására megnő a keringésbe kerülő extracelluláris vezikulák mennyisége. Kutatásunkban azt a célt tűztük ki, hogy akut, intenzív fizikai terhelés hatását vizsgáljuk az extracelluláris vezikulák mennyiségére, melyeket humán vénás vérből izoláltunk, és elemeztünk, melyek segítségével jobban átláthatóvá válhat melyik időpontú mintavételnél mekkora mennyiségű, és milyen mérettartományba eső extracelluláris vezikula van jelen. Fiatal, egészséges, férfi önkéntesek (n=12) vettek részt a vizsgálatunkban, ahol közvetlenül a terhelés előtt, után, majd 5 és 24 órával később vettünk tőlük vért, melyből extracelluláris vezikulákat izoláltunk. Differenciál centrifugálás és méretkizárásos kromatográfia után két módszerrel mértük a kis és közepes méretű vezikulák mennyiségét a mintákban. Az eredmények hatalmas egyéni különbségeket tártak fel, és kiderült, hogy a terhelés utáni 5. óra a legmegfelelőbb időpont a vérvételre, hogy begyűjtsük az extracelluláris vezikulákat.

Kulcsszavak: extracelluláris vezikulák, magas intenzitású edzés

Introduction

Exercise has systemic effects on the body (Radak et al., 2008). However, the blood flow and the oxygen supply differ significantly among organs during exercise. In the skeletal muscle blood flow can increase by 100-fold, in the brain there is a moderate increase, while in the liver, kidney, and gastrointestinal tract the oxygen supply can easily reduce by 50% of the resting values (Teglas et al., 2020). However, it is not known whether the systemic effects of exercise are due to certain messengers, agents or as a result of cyclic change in the metabolism.

In the last decades, research data has revealed that almost all types of cells are capable of generating

Table 1. Average age, body weight, body height and peak power of participants
1. táblázat. Résztvevők átlag életkora, testtömege, testmagassága és csúcsteljesítménye

Gender	N	Mean age (years)	±S.D.	Mean body weight (kg)	±S.D.	Mean body height (cm)	±S.D.	Mean Peak Power (W)	±S.D.
Men	12	26.75	2.60	79.25	8.25	180.51	7.40	1 257.66	202.15

vesicles inside the cell bodies, filling them with different cargo and release these particles into the circulation. These extracellular vesicles (EVs) then can be classified according to the size of vesicles, or according to origin or function. According to the statement paper of the International Society for Extracellular Vesicles, it is strongly suggested to refer to EVs according to their size such as ("small EVs" (sEVs) and "medium/large EVs" (m/lEVs), with ranges defined, for instance < 100 nm or < 200 nm [small], or > 200 nm [large and/or medium]) (Ridger et al., 2017). The isolation of sEVs is crucial to understanding their mechanisms.

Isolation is one of the most difficult parts of EV research; due to the huge number of influencing factors. Inappropriate isolation readily leads to false results. Isolation strategies typically used include centrifugation, ultrafiltration, and size-exclusion chromatography. The cargo of EV can be protein, lipid, RNA, microRNA or even DNA fragments.

It has been shown that physical exercise results in an increased volume of EV in the circulation (Brahmer et al., 2020), however we do not know much about the origin, cargo and the time-line of EV generation following different types of exercise. Our laboratory has a long-term interest in studying the molecular effects of physical exercise, and we decided to study the role of EVs in exercise-associated adaptation. The first part of the investigation is to set up appropriate methods to identify EVs after bouts of exercise. Therefore, the aim of the present investigation was to isolate EVs after high intensity exercise from the blood samples of young volunteers before, and at three time points after the exercise.

Material and Methods

The study was conducted in accordance with the Declaration of Helsinki for Human Research. The Ethics Committee of the Medical Research Council of Hungary (ETT-TUKEB, IV/359-2/2020/EKU) approved all experiments with human samples. Participants volunteered for the study; they received detailed written information beforehand, a statement of consent and a questionnaire on personal data was completed on paper (**Table 1.**). The data was processed anonymously.

Participants

Twelve young, healthy, male volunteered for our pilot study. They ranged between 21 – 31 years of age.

Wingate test

The Wingate test was selected as the exercise protocol. Prior to, and after the Wingate test, blood pressure, pulse, height, body weight, and lactate levels of blood from the earlobes were measured for all subjects. The Wingate test was performed on a Monark Ergomedic 894E bicycle ergometer under medical supervision. In this study we discovered that we typically find the highest power measured in 30 seconds, the average power, and the fatigue index indicators, which we can use to evaluate the anaerobic power and the anaerobic capacity.

High Intensity Interval Training (HIIT)

HIIT was performed the next day after the Wingate test at the Research Center for Molecular Exercise Science (Budapest, Hungary) under medical supervision. Subjects warmed up for 5 minutes on a bicycle ergometer. After the warm-up, the resistance was increased to 3.5 times of their body weight in watt for 40 seconds, followed by 2 minutes of rest (25 W). This was done over a total of 6 cycles, followed by a five-minute cool-down.

Preparation of blood plasma

Samples were collected at the Research Center for Molecular Exercise Science (Budapest, Hungary) by a qualified nurse. Venous blood samples (9 mL) were collected from the cubital vein of twelve healthy male volunteers without any known acute or chronic diseases into two Anticoagulant Citrate Dextrose-A (ACD-A)-containing tubes via the BD Vacutainer blood collection system. Human subjects were between 21 and 31 years old and were not taking any medication at the time of sampling. Blood samples were preceded by 12 hours of starvation and a low-fat meal. Blood samples were taken at 4 time points (before the HIIT exercise, directly after, 5 hours later, 24 hours later). Cellular components were eliminated with centrifugation (two times 2,500 g, 24°C, 15 min), a supernatant (platelet-free plasma) was loaded into microcentrifuge tubes, and put into -80 °C.

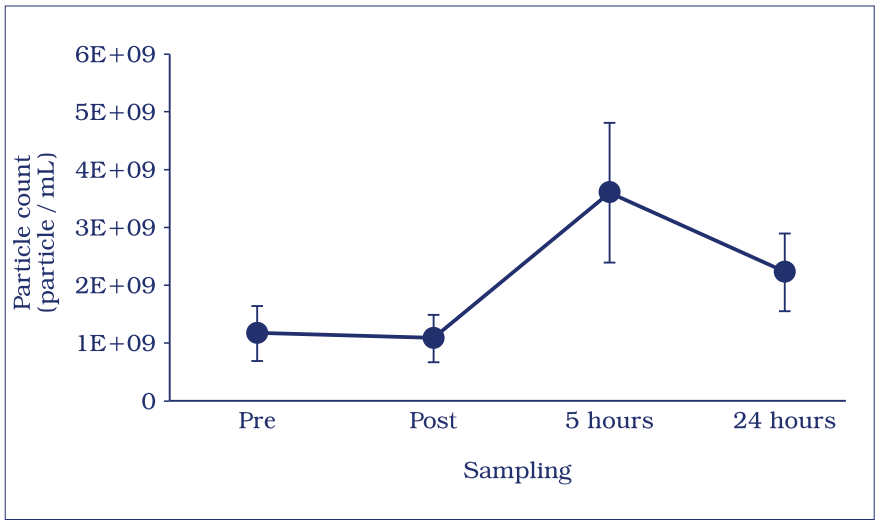

Figure 1. Concentration of medium-sized extracellular vesicles (mEVs) with ZetaView
The amount of medium-sized extracellular vesicles (mEVs) measured with the ZetaView instrument did not show a significant change with a single bout of exercise
1. ábra. ZetaView készüléken mért közepes méretű extracelluláris vezikula koncentráció
A közepes méretű extracelluláris vezikulák (mEVs) mennyiségüket ZetaView készülékkel megmérve nem mutattak szignifikáns változást a kezelés hatására

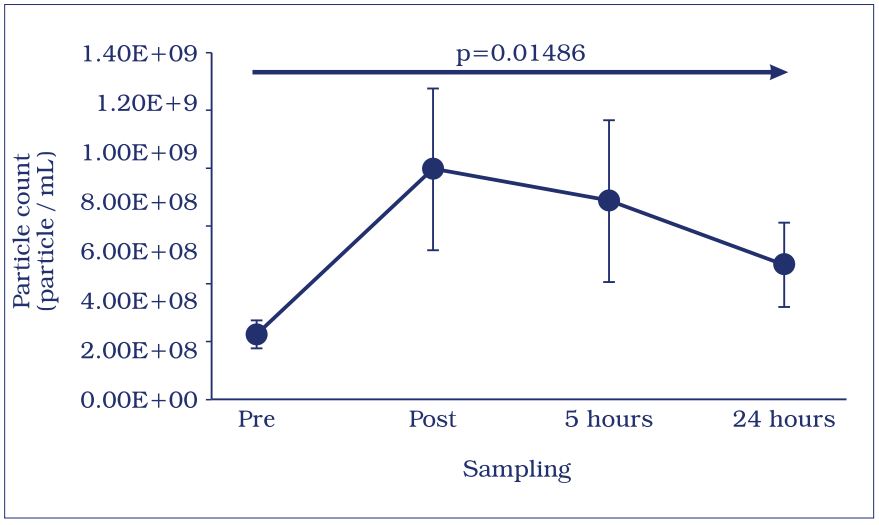

Figure 2. Concentration of medium-sized extracellular vesicles (mEVs) with qNano
There was a significant change in the concentration of medium-sized extracellular vesicles (mEVs) measured on the qNano instrument
2. ábra. qNano készüléken mért közepes méretű extracelluláris vezikula koncentráció
A közepes méretű extracelluláris vezikulák (mEVs) qNano készüléken mért koncentrációjának vizsgálatánál volt szignifikáns változás

Extracellular vesicle isolation with Size-exclusion chromatography (SEC) and differential centrifugation

The platelet free plasma (PFP) samples (3.5 ml) were diluted 2× with a 0.2-0.1 μm filtered NaCl-Hepes buffer and were filtered through a 0.8 μm sterile CA syringe filter by hydrostatic pressure to remove remaining platelets and apoptotic bodies. Seven ml of 0.8 μm filtered and 2× NaCl-Hepes diluted PFP samples were centrifuged at 18,000 g, 18°C for 20 min to remove medium-sized extracellular vesicles (mEVs). After the supernatant (small extracellular vesicles (sEVs)) was carefully aspirated from the pellet (mEVs), it was washed with NaCl-Hepes buffer at 18,000 g and 18°C for 20 min. Supernatant was gravity filtered through 0.2 μm sterile CA syringe filter and loaded into Amicon Centrifugal 4ml 100K tubes, which were centrifuged at 3,000 g, 24°C for 30 min. The ultrafiltrated samples (1 ml) were loaded onto qEVoriginal 35 nm pore size SEC columns. SEC was performed as described in the manufacturer's manual. Samples were eluted with NaCl-Hepes buffer. Seven fractions (500μl) were collected from the column; and we combined 5 fractions into a pooled one.

Size distribution and concentration of EVs

1. ZetaView Z- Nanoparticle Tracking Analysis (NTA) instrument

Particle size distribution and concentrations were measured on a ZetaView Z- Nanoparticle Tracking Analysis (NTA) instrument (Particle Metrix). Different dilutions were made to measure the size distribution and concentrations of mEVs, and the sEVs before and after ultracentrifugation.

2. Izon qNano instrument

Size distribution and concentration of mEVs and sEVs were determined by using a qNano instrument (IZON Science, Oxford, United Kingdom). Dilutions were prepared in 0.2-0.1 μm filtered NaCl-Hepes from each EV fraction (mEVs, sEVs) and measured by qNano. Results were evaluated using the software IZON Control Suite 3.2.

Spectrophotometry

Protein of non – vesicular origin was measured with a NanoDrop 1000 instrument (Thermo Fisher Scientific, Waltham, Massachusetts, United States of America). The sample's absorbance was measured on a 280 nm wavelength. NaCl-Hepes was the blank and 1.5 μl samples were used for this measurement.

Ultracentrifugation

The pooled fraction (sEVs) was pelleted by ultracentrifugation (UC) at 100,000 g, 4 °C for 70 min. After ultracentrifugation, the pellet (sEVs) was suspended in a 50 μl of NaCl-Hepes buffer and measured back with ZetaView.

Statistical analysis

The Statistica 13.2 program was used for all statistical analyses. First, a normality test was performed on the data using the Shapiro-Wilk test. Because p values were below 0.05 during the analysis, the data did not show a normal distribution, therefore, a non-parametric Friedman ANOVA & Kendall's concordance test was performed to determine the differences. Using this test, we compared the dependent samples measured at several points and in two of the four cases we found a significant change as a result of the single bout of exercise. Statistical significance was established at P=0.05.

Results

Huge individual differences were found in EV concentration following the HIIT exercise. The **Figure 1.** shows the volume change of medium sized EVs, measured by the ZetaView Z- Nanoparticle Tracking Analysis instrument before and at three different time points after the HIIT exercise. Statistical evaluation revealed a significant increase in the concentration of EVs 5 and 24 hrs after the exercise. When we checked the size distribution and concentration of medium sized EVs by the qNano instrument, it turned out that a significant peak of elevation of EVs was measured right after the exercise. Medium-sized EVs' concentration although on a less significant level was still higher than the normal value at the 5 and 24 hrs time points after the exercise test (**Figure 2.**).

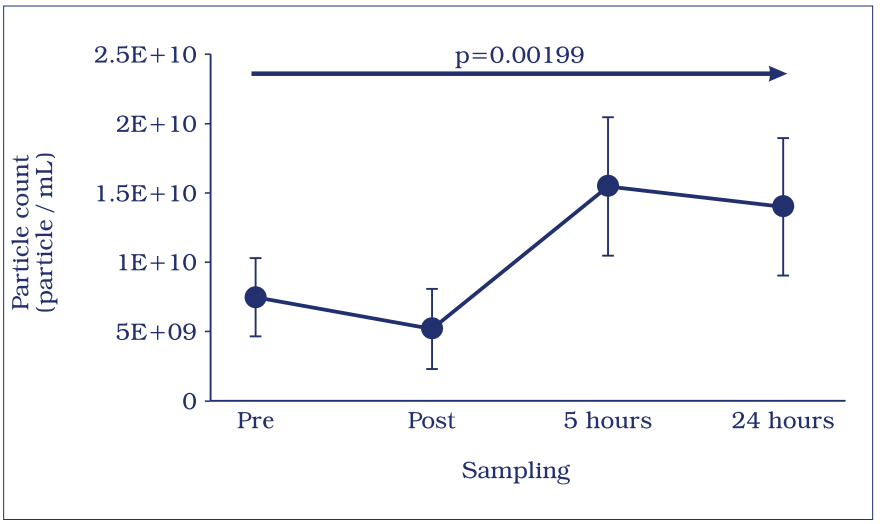

Figure 3. Concentration of small extracellular vesicles (sEVs) with ZetaView after SEC
There was a significant change in the results of the fractions of small extracellular vesicles (sEVs) obtained after size-exclusion chromatography on a ZetaView instrument, where the vesicle concentration was examined

3. ábra. ZetaView készüléken mért kis méretű extracelluláris vezikula koncentráció SEC után
A kisméretű extracelluláris vezikulák (sEVs) méretkizárásos kromatográfia után kapott frakcióinak ZetaView készüléken mért eredményeinél szignifikáns változás volt, ahol a vezikula koncentrációt vizsgáltuk

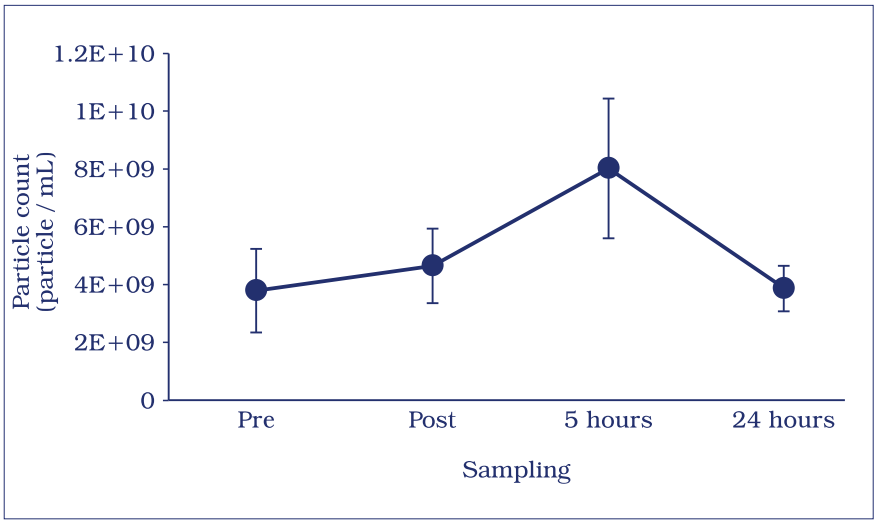

Figure 4. Concentration of small extracellular vesicles (sEVs) with ZetaView after UC
The results of the fractions of small extracellular vesicles (sEVs) obtained after ultracentrifugation and ímeasured on a ZetaView instrument showed no significant change in concentration

4. ábra. ZetaView készüléken mért kis méretű extracelluláris vezikula koncentráció UC után
A kisméretű extracelluláris vezikulák (sEVs) ultracentrifugálás után kapott frakcióinak ZetaView készüléken mért eredményeinél nem volt szignifikáns változás a koncentrációban

Table 2. P-values of the statistical analysis
2. táblázat. A statisztikai analízis p-értékei

Measurements	p-values
mEVs with ZetaView	0.25236
mEVs with qNano	0.01486
sEVs with ZetaView after SEC	0.00199
sEVs with ZetaView after UC	0.51679

For the evaluation of sEVs we used the ZetaView system after size-exclusion chromatography without (**Figure 3.**) and with ultracentrifugation (**Figure 4.**). **Figure 3.** shows that small EVs concentration increased significantly 5 and 24 hrs after exercise. Due to a technical problem, we could not use the qNano system at sEVs.

The following table (**Table 2.**) shows the p values of the statistical analysis.

Discussion

In the present investigation we studied the methodology of medium and small EVs before and at three time points after HIIT exercise. One of the important observations of this study were the huge individual differences in the amount of EV concentration, which significantly influenced the selection of statistical methods with which we analyzed the obtained data. We tested different approaches to isolate medium and small size EVs. Although both the ZetaView and the qNano instruments are often used in studies focusing on EVs, we found quite different data outputs with these instruments. In addition, we discovered that the qNano system could not be used efficiently in our case for the isolation of small EVs. This pilot study revealed that both the ZetaView and qNano instruments can be used to successfully select medium EVs following a single bout of exercise. Moreover, it appears that HIIT exercise results in a significantly elevated concentration of medium size EVs 5 and 24 hrs following exercise protocol. The immediate response can be measured only by the qNano method. On the other hand, in case of small EVs the significant increase appeared 5 hrs after exercise bouts. From this data it cannot be concluded that medium and small EV secretion is different following high intensity exercise. It would be interesting to identify the main organs doing the secretion of EVs and to find out the differences or similarities of the cargo of medium and small EVs. We are presently working on this project.

To our knowledge this could be one of the first studies which aims to investigate the time-course of EV release following high intensity exercise. We believe that to know the time course and the cargo of different sized EVs would allow us to better understand the systemic adaptation to physical exercise.

Acknowledgements

The present study was supported by UNKP grant given to GB.

References

Brahmer, A., Neuberger, E.W.I., Simon, P., Kramer-Albers, E.M. (2020): Considerations for the analysis of small extracellular vesicles in physical exercise. *Frontiers in Physiology*, **11:** 576150.

Radak, Z., Chung, H.Y., Goto, S. (2008): Systemic adaptation to oxidative challenge induced by regular exercise. *Free Radical Biology & Medicine*, **44:** 153-159.

Ridger, V.C., Boulanger, C.M., Angelillo-Scherer, A., Badimon, L., Blanc-Brude, O., Bochaton-Piallat, M.L., Boilard, E., Buzas, E.I., Caporali, A., Dignat-George, F., Evans, P.C., Lacroix, R., Lutgens, E., Ketelhuth, D.F.J., Nieuwland, R., Toti, F., Tunon, J., Weber, C., Hoefer, I.E. (2017): Microvesicles in vascular homeostasis and diseases: Position Paper of the European Society of Cardiology (ESC) Working Group on Atherosclerosis and Vascular Biology. *Thrombosis and homeostasis*, **117:** 1296-1316.

Teglas, T., Abraham, D., Jokai, M., Kondo, S., Mohammadi, R., Feher, J., Szabo, D., Wilhelm, M., Radak, Z. (2020): Exercise combined with a probiotics treatment alters the microbiome, but moderately affects signaling pathways in the liver of male APP/PS1 transgenic mice. *Biogerontology*, **21:** 807-815.