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RESEARCH ARTICLE

Determination of possible microRNA-markers of cobalt abuse by real-time qPCR using hypoxia signaling pathway panels

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Abstract

Objectives. *Cobalt mimics the state of hypoxia to prevent degradation of the alpha subunit of hypoxia-inducible factor, resulting in an increase in blood oxygen capacity and endurance. Athletes can use this property to gain competitive advantage. Nowadays, direct methods of inductively coupled plasma mass spectrometry and liquid chromatography-tandem mass spectrometry are used to determine total cobalt levels in the body. However, the World Anti-Doping Agency is yet to establish a maximum allowable threshold concentration of this element in biofluids. The lack of clear identification criteria complicates the interpretation of the obtained results for the purposes of doping control. In this regard, the present work proposes a new approach for the indirect determination of possible cobalt abuse based on changes in the expression levels of miRNAs involved in the regulation of hypoxia signaling pathways. Here, the aim is to identify possible microRNA markers whose expression does not depend on exercise-induced hypoxia, but changes markedly when taking cobalt preparations.*

Methods. *MicroRNA isolation was performed from blood plasma samples using the PAXgene Blood miRNA Kit. Quantitative real-time polymerase chain reaction (PCR) was performed on CFX96 Bio-Rad (USA) analyzer using miScript® SYBR® Green PCR Kits and panels for studying the expression profiles of mature microRNAs of the hypoxia signaling pathway miScript® miRNA PCR Array.*

Results. *Based on the statistical analysis of the data, it was found that the expression of hsa-miR-15b-5p in the blood plasma of the subjects does not depend on physical activity, but increases when taking cobalt preparations.*
Conclusions. *The difference in expression levels during anaerobic exercise-induced hypoxia and cobalt-induced hypoxia makes hsa-miR-15b-5p a potential candidate to be a marker of erythropoiesis-stimulating agent abuse.*

Keywords: *cobalt, qPCR-RT, microRNA, doping control, erythropoiesis stimulants*

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НАУЧНАЯ СТАТЬЯ

Определение возможных микроРНК-маркеров злоупотребления препаратами кобальта методом ПЦР в реальном времени с использованием панелей сигнального пути гипоксии

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Аннотация

Цели. *Кобальт имитирует состояние гипоксии, препятствуя деградации альфа-субъединицы гипоксия-индуцируемого фактора, что приводит к увеличению кислородной емкости крови и может использоваться спортсменами в качестве допинга для получения конкурентных преимуществ. На сегодняшний момент для определения общего кобальта в организме используют прямые методы масс-спектрометрии с индуктивно связанной плазмой, жидкостной хроматографии-тандемной масс-спектрометрии, однако Всемирным антидопинговым агентством не установлена максимально допустимая пороговая концентрация этого элемента в биожидкостях. Отсутствие четких критериев идентификации осложняет интерптацию полученных результатов. В связи с этим, в данной статье впервые предлагается подход по косвенному определению возможных злоупотреблений кобальтом для целей допинг-контроля, основанный на изменении уровней экспрессии микроРНК, задействованных в регуляции сигнального пути гипоксии. Цель исследования заключалась в поиске возможных микроРНК-маркеров, экспрессия которых не зависит от гипоксии, вызванной физическими нагрузками, но заметно изменяется при приеме препаратов кобальта.*

Методы. *Выделение микроРНК из образцов плазмы крови проводили при помощи набора PAXgene Blood miRNA Kit. Количественную полимеразную цепную реакцию в реальном времени проводили на амплификаторе CFX96 Bio-Rad (США) с помощью наборов miScript® SYBR® Green PCR Kit и панелей для исследования профилей экспрессии зрелых микроРНК сигнального пути гипоксии Hypoxia Signaling Pathway miScript® miRNA PCR Array.*

Результаты. *На основании статистического анализа данных было установлено, что экспрессия hsa-miR-15b-5p в плазме крови испытуемых не зависит от физической нагрузки, но возрастает при приеме препаратов кобальта.*

Выводы. *Разница в уровнях экспрессии при гипоксии, вызванной анаэробной физической нагрузкой, и имитацией гипоксии за счет применения кобальта делает hsa-miR-15b-5p потенциальным претендентом на роль маркера злоупотребления данным эритропоэз-стимулирующим агентом.*

Ключевые слова: *кобальт, qPCR-RT, микроРНК, допинг-контроль, стимуляторы эритропоэза*

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INTRODUCTION

Up until the 1980s, cobalt(II) compounds were often used to treat various forms of anemia [1–3]. The mechanism of their action is associated with the activation of the hypoxia-inducible transcription factor (HIF), which leads to an increase in the expression of the erythropoietin (EPO) gene, a key regulator of the production of red blood cells (erythrocytes), as well as an increase in the oxygen capacity of the blood [4–6]. However, with a more detailed study of the serious side effects resulting from the intake of large doses of cobalt, which include acute poisoning, cardiac arrhythmias and mental disorders, as well as stimulation of carcinogenesis [7–10], their use for clinical purposes tended to be replaced by epoetin (recombinant human erythropoietin)-based drugs [11]. Despite this, some professional ahtletes continue to use various cobalt-containing supplements and even cobalt(II) chloride suspension to improve exercise tolerance and aerobic endurance, which can provide a competitive advantage. Cobalt is included in the World Anti-Doping Agency (WADA) Prohibited List[1] and is classified as a "hypoxia-inducible factor activator" in accordance with Article S2 "Peptide Hormones, Growth Factors, Like Substances and Mimetics."

As of today, only a few anti-doping laboratories use direct methods for the determination of cobalt: inductively coupled plasma mass spectrometry (ICP-MS) [12–15], liquid chromatography with tandem mass spectrometry [16, 17], or a combination of them. However, since the majority do not include these approaches at all in their methodological arsenal, the problem of revealing the fact of the abuse of salts of this element as a doping agent remains. In addition, WADA has not yet established the maximum allowable threshold concentration of this element in bioliquids or any identification criteria, further complicating the interpretation of the results.

[1] WADA Prohibited List 2022. URL: https://www.wada-ama.org/sites/default/files/resources/files/2022list_final_en.pdf. Accessed November 10, 2022.

A number of works [15, 18, 19] use an approach based on comparing the ratio of the level of vitamin B12, an immunochemically measured source of cobalt present in the body in its natural state, in tandem with the concentration of total cobalt as measured by ICP-MS. Despite the possibility of using this promising approach to indirectly detect the presence of cobalt salts in the body, it is rather laborious for large-scale screening studies. In addition, several scientific articles are devoted to the study of erythropoietic effects when taking low doses of inorganic cobalt [4, 20]. The program of the hematological module of the athlete's biological passport (ABP)[2] permits the indirect determination of possible manipulations with blood and the fact of taking some erythropoiesis-stimulating agents (ESA) due to a sharp increase in hemoglobin concentration and a decrease in the percentage of reticulocytes, as well as analysis of a number of other parameters. However, it remains impossible to establish whether the changes in the blood are the result of high-altitude training of athletes or the result of taking illegal preparations.

In connection with the above, there is a need to develop a new analytical approach to indirectly determine the abuse of cobalt salts based on changes in microRNA expression profiles as a result of taking drugs containing cobalt. Such changes in the profiles of small non-coding blood RNAs can act as earlier highly sensitive markers of response to a physiological or pathological stimulus than biochemical ones [21–24].

MATERIALS AND METHODS

Research samples

For the study, blood plasma samples were taken from volunteers aged 22–35 years (n = 10) leading an active lifestyle (control group), as well as blood plasma from athletes participating in a race-walking discipline (group 1) and blood plasma from the same volunteers after taking the dietary supplement Cobalt DS® (*Dr. Skalny*, Russia) (group 2) for 20 days in an amount 4 times the recommended dosage (1 tablet contains 200 mcg of cobalt aspartate) after a meal. To obtain plasma, samples of venous blood taken on an empty stomach were centrifuged for 10 min at 20°C at a speed of 1500 rpm in a Rotixa 50 RS centrifuge (*Hettich Zentrifugen*, Germany). The entire plasma volume was collected and frozen at −20°C until analysis could be performed.

After being informed about the goals of the experiment, volunteers provided written consent to use their biological material for scientific purposes. The study was carried out in accordance with the principles contained in the Declaration of Helsinki[3] and was approved by the local ethics committee of the Moscow Scientific and Practical Center for Medical Rehabilitation, Restorative and Sports Medicine of the Moscow Health Department. Athletes' blood plasma samples were taken in accordance with paragraph 5.3.12.2 of the International Standard for Laboratories[4] and paragraph 6.3 of the World Anti-Doping Code from the archive of the laboratory. Volunteer blood samples were taken according to the ABP sampling rules in BD Vacutainer® Plus EDTA (K2EDTA) vacuum tubes (*Becton Dickinson*, USA) for hematological studies.

Isolation of miRNAs from blood plasma samples

MicroRNAs were isolated from 2 mL of plasma using the PAXgene Blood miRNA Kit (*PreAnalytiX*, Switzerland) according to the manufacturer's protocol with some modifications. Changes to the standard method were necessary due to the fact that blood was collected not in specialized PAXgene tubes (*QIAGEN*, Germany), but in BD Vacutainer® Plus tubes (*Becton Dickinson*, USA). The following modifications were used: 2 mL of plasma was mixed with 2 mL of a denaturing buffer solution (2.7 M guanidine thiocyanate, 1.3 M ammonium thiocyanate, 100 mM sodium acetate, 5 mM EDTA, pH 4.0), incubated at 20–25°C for 20 min, centrifuged for 10 min at 14000g, the precipitate was washed twice with 2 mL of deionized water. After the second washing, the precipitate was dissolved in 350 μL of the BM1 buffer solution from the above kit, then miRNAs were isolated according to the manufacturer's protocol, miRNAs were washed off the columns with two volumes of 40 μL of deionized water.

[2] WADA Athlete biological passport operating guidelines. URL: https://www.wada-ama.org/sites/default/files/resources/files/guidelines_abp_v8_final.pdf. Accessed November 15, 2022.

[3] Declaration of Helsinki of the World Medical Association. URL: https://www.wma.net/policies-post/wma-declaration-of-helsinki-ethical-principles-for-medical-research-involving-human-subjects/. Accessed November 15, 2022.

[4] International Standard for Laboratories URL: https://www.wada-ama.org/sites/default/files/resources/files/isl_2021.pdf. Accessed November 15, 2022.

MicroRNA concentration was measured using a Fluo 100 fluorimeter (*Hangzhou Allsheng Instruments*, China) according to the instructions. The isolated miRNA was stored at −20°C for further use.

Conducting reverse transcription and quantitative polymerase chain reaction (PCR)

The reverse transcription reaction was carried out using the miScript® II RT Kit (*QIAGEN*, Germany) using 5×miScript HiSpec Buffer according to the manufacturer's protocol on a C1000 Touch Thermal Cycler (*Bio-Rad*, USA), 200 ng of miRNA were taken for the reaction, the final volume of the reaction mixture is 20 μL. The resulting cDNA was stored at −20°C. Before performing quantitative PCR, cDNA was diluted by adding 200 μL of deionized water. Quantitative PCR with real-time detection was performed on a CFX96 DNA amplifier (*Bio-Rad*, USA) using miScript® SYBR® Green PCR Kits (*QIAGEN*, Germany) and panels for studying the expression profiles of mature miRNAs of the hypoxia signaling pathway Hypoxia Signaling Pathway miScript® miRNA PCR Array (*QIAGEN*, Germany). PCR program: initiation 95°C, 15 min, 40 cycles (94°C – 15 s, 55°C – 30 s, 70°C – 30 s). Reverse transcription and PCR controls and reference genes are included in the panel.

Statistical processing of results

Statistical processing of the results was performed using CFX Maestro Software (*Bio-Rad*, USA) and GeneGlobe online software (*QIAGEN*, Germany). The experimental data were combined into one study. In the work, the normalized miRNA expression (ΔCt) was determined; normalization was performed for reference RNAs contained in the used Hypoxia Signaling Pathway miScript® miRNA PCR Array panel (*QIAGEN*, Germany). According to the coefficients of variation (CV) and geometric mean values (*M*-Value), SNORD68 (CV 0.3690, *M* 0.8174), SNORD95 (CV 0.0282, *M* 0.5652), SNORD96a (CV 0.3951, *M* 0.8783) were selected as references with average stability values CV 0.2641 and *M* 0.7537, because for heterogeneous samples, it is recommended to use reference RNAs with CV < 0.5 and *M* < 1.0. When calculating the relative normalized miRNA expression (ΔΔCt), the averaged ΔCt values of the experimental group versus the control group were used. The values $\Delta\Delta Ct < 2^{-2}$ and $2^{2} < \Delta\Delta Ct$ ($p < 0.01$) were considered significant.

RESULTS AND DISCUSSION

ESAs are more often used in sports focused on prolonged physical activity and long distances (athletics, cycling, race walking, biathlon, etc.) to improve the supply of oxygen to the body's organs and tissues and increase endurance. However, it should be taken into account that athletes develop an exercise-induced state of hypoxia, which in itself contributes to the activation of HIF and the start of the process of erythropoiesis. Therefore, the detection of a difference in the markers of the hypoxia signaling pathway during anaerobic exercise and the use of cobalt prohibited by WADA is very important for modern doping control.

In most of the studied samples, miRNA expression either changed insignificantly or the reliability of the obtained data was below $p = 0.01$. Figure 1 shows a comparison of the levels of expression of microRNAs of the hypoxia signaling pathway circulating in plasma in experimental groups 1 (athletes) and 2 (volunteers taking dietary supplements Cobalt DS®).

The abscissa shows the logarithm (Log 2) of the ratio of the miRNA expression level in group 2 to the expression level of the same miRNA in group 1. The solid vertical line corresponds to the absence of changes in the expression level, while the dotted vertical lines correspond to a decrease or increase in the expression level in group 2 compared to group 1 to 4 times.

The negative logarithm (−Log 10) of the *p*-value is plotted along the ordinate axis, reflecting the significance of the obtained results. The solid horizontal line corresponds to $p = 0.01$. Thus, changes in the level of expression shown on this graph above the solid horizontal line are statistically significant, and those shown below it are not statistically significant.

Based on the analysis of data on the levels of microRNAs circulating in the plasma in the three studied groups, nine microRNAs were selected, whose expression is stably determined in all samples, as well as changing under conditions of hypoxia, both caused by aerobic exercise and by the intake of dietary supplements containing cobalt (Table). These include miR-135a-5p, miR-15b-5p, miR-199b-5p, miR-200b-3p, miR-203a-3p, miR-204-5p, miR-26a-5p, miR-449a, miR-504-5p (Fig. 2).

Figure 2 shows that seven (miR-135a-5p, miR-199b-5p, miR-200b-3p, miR-203a-3p, miR-204-5p, miR-449a, miR-504-5p) out of nine selected miRNAs in athletes are expressed at a higher level than in volunteers who do not participate in sporting activities. After taking Cobalt DS®, their expression level also increased, but was significantly lower than in athletes.

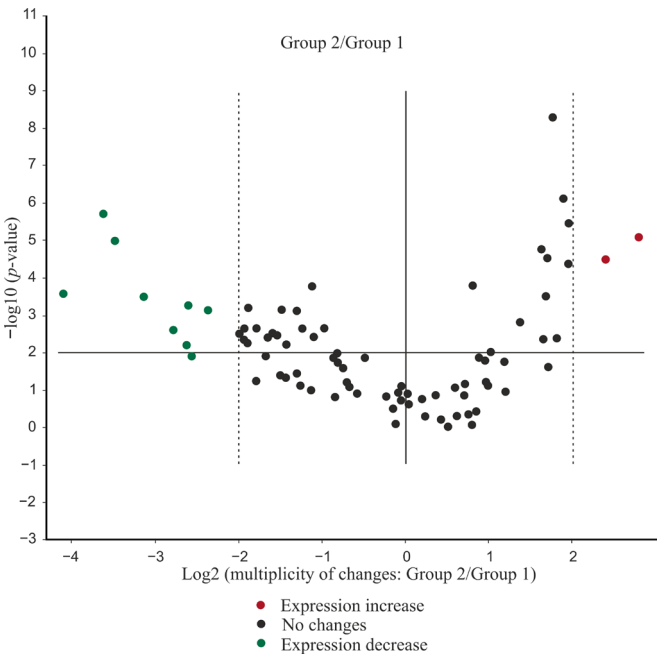

Fig. 1. Comparison of expression levels of microRNAs of the hypoxia signaling pathway circulating in plasma in experimental groups 1 (athletes) and 2 (volunteers taking dietary supplements Cobalt DS®).

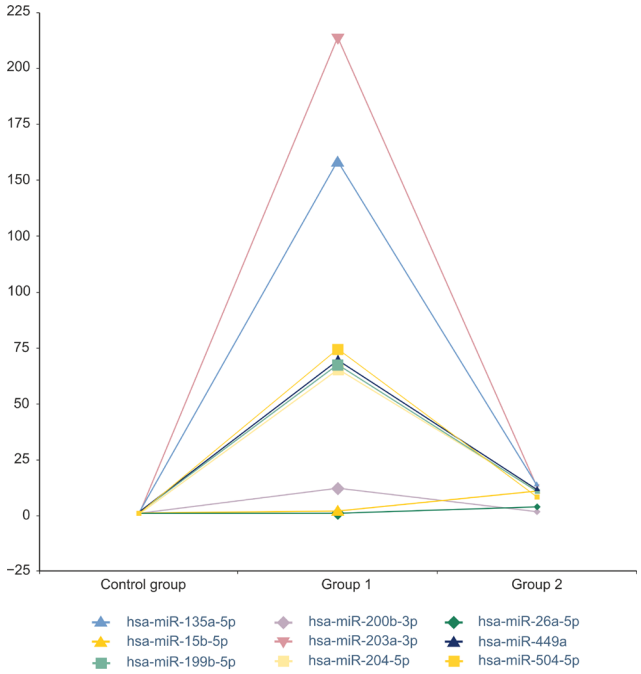

Fig. 2. Line diagram showing changes in the expression of selected circulating miRNAs in different groups. Based on the data presented in the figure, it can be seen that in group 2, there is an increase in the expression of all miRNAs, except for miR-15b-5p, whose expression remains unchanged, but when taking dietary supplements Cobalt DS®, it increases.

For our purposes, the use of these seven miRNAs as markers of ESA application will be practically difficult, since this is possible only by tracking their expression levels over time, similar to the ABP hematological module program. MiR-26a-5p levels were observed to vary slightly between groups.

Sports medicine professionals who work directly with athletes may also be interested in hsa-miR-135a-5p and hsa-miR-203a-3p. The level of these microRNAs in the blood plasma is 11.2 (hsa-miR-135a-5p) and 17.1 (hsa-miR-203a-3p) times higher in athletes than in volunteers, respectively (Table). Comparison of microRNA data levels in athletes with different levels of training and/or in athletes of different sports would suggest the possibility of their use as markers of physical fitness in general or markers of resistance to anaerobic stress, in particular. From a scientific point of view, it is interesting to determine whether a high level of miRNA expression is genetically determined. Studies by Guo *et al.* [25] showed that has-miR-135a-5p reduces the number of apoptotic myocardial cells and inflammation after myocardial infarction by inhibiting the expression of inflammatory proteins: TNF-α, IL-1β, and IL-6. Hsa-miR-135a-5p also directly interacts with the inhibitor of hypoxia-inducible factor 1 alpha (HIF1AN) to reduce its expression and thus indirectly increase the expression of genes responsible for osteogenesis processes [26]. By screening online databases [27], it was predicted and then experimentally confirmed that has-miR-135a-5p can potentially be involved in the regulation of TRPC1 proteins (transient receptor potential calcium channels (TRPC)). TRPC1 is a differential regulator of hypoxia-mediated events. Blocking TRPC1 reduces hypoxia-induced autophagy, which promotes cell survival under hypoxic conditions. Hsa-miR-203a-3p, according to [28], prevents the development of ischemic stroke by suppressing apoptosis, oxidative stress, and inflammation.

The hsa-miR-15b-5p seems to be the most interesting of the 84 studied microRNAs since its expression in athletes (group 1) remains unchanged, as in the volunteers of the control group; after taking Cobalt DS®, its expression is significantly higher (group 2). Previously, hsa-miR-15b-5p was studied in connection with the search for markers of resistance to mountain sickness, which causes pulmonary edema and cerebral edema under hypoxic conditions at altitudes of more than 2500 m above sea level [29]. Subjects with a higher initial level of hsa-miR-15b-5p tolerated hypoxia conditions more easily and did not show signs of the development of altitude sickness.

Table. The ratio of expression levels of circulating miRNAs in group 2 (volunteers taking dietary supplements Cobalt DS®) to that in group 1 (athletes), group averages

No.	01	02	03	04	05	06	07	08	09	10	11	12
A	hsa-let-7a-5p **3.38**	hsa-let-7b-5p **2.02**	hsa-let-7c-5p **2.59**	hsa-let-7d-5p **3.86**	hsa-let-7e-5p **3.24**	hsa-let-7f-5p **3.69**	hsa-let-7g-5p **3.09** A	hsa-let-7i-5p **1.50**	hsa-miR-101-3p **-2.48**	hsa-miR-103a-3p **-1.06** B	hsa-miR-107 **-5.16**	hsa-miR-10b-5p **-3.47** B
B	hsa-miR-122-5p **-3.16**	hsa-miR-125a-5p **1.97** B	hsa-miR-125b-5p **-1.81**	hsa-miR-130a-3p **1.74**	hsa-miR-130b-3p **1.84**	hsa-miR-135a-5p **-11.16**	hsa-miR-138-5p **-2.18** B	hsa-miR-141-3p **-2.18** B	hsa-miR-146a-5p **-1.17** B	hsa-miR-146b-5p **1.01** B	hsa-miR-148a-3p **-1.11** B	hsa-miR-148b-3p **-1.63** B
C	hsa-miR-150-5p **1.28**	hsa-miR-155-5p **1.14** B	hsa-miR-15a-5p **-2.80**	hsa-miR-15b-5p **6.89**	hsa-miR-16-5p **1.41**	hsa-miR-17-5p **1.94** B	hsa-miR-181a-5p **-1.80** B	hsa-miR-181b-5p **3.50**	hsa-miR-181c-5p **-3.18**	hsa-miR-184 **-2.82**	hsa-miR-186-5p **-1.76**	hsa-miR-188-5p **-2.48**
D	hsa-miR-191-5p **3.86** A	hsa-miR-192-5p **1.62** B	hsa-miR-195-5p **1.68**	hsa-miR-199a-5p **1.18** B	hsa-miR-199b-5p **-6.19**	hsa-miR-19a-3p **-2.40** B	hsa-miR-200a-3p **-3.84**	hsa-miR-200b-3p **-6.87**	hsa-miR-203a-3p **-17.09**	hsa-miR-204-5p **-6.07**	hsa-miR-205-5p **-3.73**	hsa-miR-20a-5p **2.29** B
E	hsa-miR-20b-5p **-1.76**	hsa-miR-210-3p **-1.50** B	hsa-miR-215-5p **-2.69**	hsa-miR-21-5p **1.52**	hsa-miR-221-3p **-1.04** B	hsa-miR-22-3p **-3.03**	hsa-miR-224-5p **-2.15**	hsa-miR-23a-3p **3.12** A	hsa-miR-23b-3p **1.34** B	hsa-miR-24-3p **-1.04** B	hsa-miR-26a-5p **5.23** A	hsa-miR-26b-5p **3.25** A
F	hsa-miR-27a-3p **-1.40**	hsa-miR-29b-3p **1.73** B	hsa-miR-30b-5p **-3.45**	hsa-miR-30e-5p **-1.60** B	hsa-miR-31-5p **-3.82**	hsa-miR-320a **1.62**	hsa-miR-324-5p **1.02** B	hsa-miR-331-3p **-1.09** B	hsa-miR-335-5p **-2.70**	hsa-miR-34a-5p **-1.96**	hsa-miR-378a-3p **-1.69**	hsa-miR-429 **-12.29**
G	hsa-miR-449a **-5.90**	hsa-miR-451a **1.79**	hsa-miR-491-5p **-2.35**	hsa-miR-504-5p **-8.79**	hsa-miR-7-5p **2.26**	hsa-miR-877-3p **-3.69**	hsa-miR-92a-3p **1.92**	hsa-miR-935 **-2.91**	hsa-miR-93-5p **3.20** A	hsa-miR-9-5p **-2.20** B	hsa-miR-98-5p **-1.02** B	hsa-miR-99a-5p **-3.94**

Note: A – the expression of this microRNA is highly variable among individuals, regardless of which group they belong to. Most likely, this microRNA cannot be used as a biological marker for the use of prohibited substances; B – the expression of this miRNA is relatively low in both control and experimental samples. In some samples, this miRNA was not detected at all. Thus, the possibility of using this microRNA as a marker for the use of ESA is also unlikely.

The clear difference in expression levels we identified between anaerobic exercise-induced hypoxia and cobalt-induced hypoxia imitation makes hsa-miR-15b-5p a potential candidate for the role of an ESA abuse marker.

CONCLUSIONS

In the course of the conducted pilot studies comparing the levels of circulating microRNAs in the blood plasma of volunteers, professional athletes involved in endurance sports, and the same volunteers after taking a dietary supplement containing cobalt, microRNA candidates were selected, whose expression was stably determined in all samples. These changed under conditions of hypoxia, both when caused by aerobic exercise and the intake of dietary supplements. Of particular interest as a possible potential marker for the indirect determination of HIF activators is

hsa-miR-15b-5p, whose expression increases markedly after 20 days of application of Cobalt DS®, but remains unchanged during prolonged exercise. To refine the obtained data, it is necessary to expand the sample of the studied samples. In view of the absence of a threshold value established by WADA for the direct determination of the concentration of total cobalt in plasma and urine, the search for new markers of ESA doping abuse is undoubtedly of practical importance for the purposes of doping control since such markers can act as additional evidence of the use of prohibited hypoxia mimetics.

Authors' contributions

P.V. Postnikov – formulation of aims and objectives, discussion of experiments and results, conducting experimental research, writing and editing the text of the article, editing the final version of the article, and preparing materials for publication;

F.V. Radus – assistance in conducting experimental research, discussion of experiments and results;

Yu.A. Efimova – discussion of experiments and results, editing the final version of the article, and preparing materials for publication;

I.V. Pronina – formulation of aims and objectives, discussion of experiments and results, conducting experimental research, writing and editing the text of the article, editing the final version of the article.

The authors declare no conflicts of interest.

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