

+Original Research Article

DIFFERENTIATION OF HEMODYNAMICS OF TOP ATHLETES DEPENDING ON HEART RATE VARIABILITY AFTER TRAINING

Aims: To predict the functional status of the cardiorespiratory system of athletes based on results of responses to exercise.

Study design: Case-control study.

Place and Duration of Study: Palace of Sports "Dynamo" in Lviv, between January and February 2016.

Methodology: 32 qualified waterpolo male athletes aged 20.6 ± 3.0 years were examined. The research included the study of physical parameters, HR and BP by using routine methods and changes of these parameters during the first 3 minutes after the Martinet Test (1 hour before training) and also the study of cardiorespiratory system using SACR before and during the first 5 minutes after training in state of relative relax in the sitting position. To assess the research results we have used the distribution-free method of statistical analysis, using which we can evaluate the Wilcoxon and Mann-Whitney criteria, and also percentile method analysis based on determining the individual assessments of each indicators that take into consideration falling in appropriate limits of percentile ranges.

Results: Hypokinetic type of hemodynamic is observed in 64% of athletes (EG_2) and in 88.2% of athletes (EG_1). According to the parameters of central hemodynamic, describing the size of the left ventricle in athletes from EG_2 , significantly greater ($p < 0.01$) is the end-diastolic volume (EDV)- $116.3(107.1;118.8) \text{ cm}^3$ and end-systolic volume (ESV)- $37.2(33.9;39.2) \text{ cm}^3$ comparing to EDV $92.5(87.0;107.6) \text{ cm}^3$ and ESV $27.1(22.4;33.7) \text{ cm}^3$ in EG_1 . Significantly larger ($p < 0.05$) was a stroke volume $78.7(72.5;79.8) \text{ cm}^3$ comparing to $64.9(61.6;77.1) \text{ cm}^3$ in EG_1 . The rate of α -factor that characterizes the BRS and predicts the effectiveness of the regulation of cardiac pump function was significantly higher ($p < 0.01$) with EG_2 : BRS_{LF} : $19.8(17.3;22.1)$ versus $10.7(8.7;17.5)$, BRS_{HF} in EG_2 : $25.4(17.0;29.7)$ comparing to $12.8(8.9;24.9)$ in EG_1 .

Conclusion: The research revealed that the mentioned features of changes in heart rate variability in the high-frequency range after training have rather accurate determinants in hemodynamic securing an athlete, which in turn can be used to predict and adequately assess the state of the athlete in the recovery period after the competition.

Keywords: central hemodynamics; heart rate response on loads; chronotropic and inotropic cardiac functioning.

1. INTRODUCTION

The study of central hemodynamics of athletes is one of the important areas of sports medicine, aimed at identifying the characteristics of the body that trains and diagnosis of preparedness. Body preparedness determines the level of fitness and characterizes the readiness of athlete to achieve high sports results. It develops under the influence of systematic and targeted training, and its level depends on the balanced interaction of many functional systems that determine the nature of adaptive capacity, hemodynamics being among the most important ones [1,2,3]. To determine the functional condition and adaptive capacities, heart rate variability, blood pressure, and central hemodynamics of athletes are recently taken into consideration [4,5,6]. Such techniques are applied in sports medicine practice when it is primarily necessary to identify prepathological changes in the body of athletes, to predict athletic performance, which is only possible with a clear understanding of applied and adaptive mechanisms that develop in the body under the influence of training activity [7,8,9]. Recognition of these mechanisms can not only determine the tolerance towards physical exercises, but also adjust training process purposefully with the use of various kinds of exercises. Determining the mechanisms of adaptation allows to monitor the impact of training loads on objective criteria of cardiorespiratory system at all times. At present the main methods of monitoring the impact of training process on the

cardiovascular system remain routine: heart rate (HR) and blood pressure (BP) controls. This is due to the failure to use modern methods of instrumental diagnostics in terms of the training process.

2. MATERIAL AND METHODS / EXPERIMENTAL DETAILS / METHODOLOGY

Modern semi-functional method of research of cardio-respiratory system (Spiroartery-cardiorhythmography (SACR)) is used to study the hemodynamic features of athletes. It allows to simultaneously register interconnected indicators characterizing the activity of the heart (first part of electrocardiogram), vessels (peripheral systolic (SBP) and diastolic (DBP) blood pressure in the middle phalanx of a finger using Penaza method, and respiratory system (according to ultrasound spirometry) [10]. The method allows to determine the volume of the influence of autonomic nervous system on heart rate (HR), blood pressure (BP), spontaneous breathing (SB). According to the data of testing HR, SBP and DBP sequences for each heartbeat and lung ventilation spectral Fourier analysis was applied, which determines the capacity of regulatory influences in different frequency ranges related to general activity, activities of suprasegmental structures and parasympathetic and sympathetic branches of autonomic nervous system (ANS) [11]. Spectral analysis is conducted in three frequency ranges: very low frequency (VLF, 0-0.04 Hz), low frequency (LF, 0.04-0.15 Hz) and high frequency (HF, 0.15-0.4 Hz), measured in absolute values of power (ms^2 - for HR, mmHg^2 - for SBP and DBP, $(\text{l} / \text{min})^2$ - for spontaneous breathing). The ratio LF / HF is used to describe vegetative balance [12]. Baroreflex sensitivity (BRS) indicators defined by spectral method and called α -factor were analyzed. α -factor was measured separately within the range of high (BRS_{HF}) and low (BRS_{LF}) frequencies [13].

The data of central hemodynamics were determined on the basis of the first part of electrocardiogram by means of method of double-phase reconstruction proposed by Kim T.N. [14].

To assess the research results we have used the distribution-free method of statistical analysis, using which we can evaluate the Wilcoxon and Mann-Whitney criteria, and also percentile method analysis based on determining the individual assessments of each indicators that take into consideration falling in appropriate limits of percentile ranges. [15,16]. The assessment of individual performance was conducted as follows: the range of <5% - expressed decrease; the range of 5 - 25% - moderate decrease; the range of 25-75% - normative; the range of 75-95% - modest increase; the range of > 95% - expressed increase.

3. RESULTS AND DISCUSSION

32 qualified waterpolo male athletes aged 20.6 ± 3.0 years were examined in regards of the impact of trainings on their bodies. The research included the study of physical parameters, HR and BP by using routine methods and changes of these parameters during the first 3 minutes after the Martinet Test (1 hour before training) and also the study of cardiorespiratory system using SACR before and during the first 5 minutes after training in state of relative relax in the sitting position. The training lasted for 2 hours and involved sessions in the pool, which was aimed at developing speed endurance. It was conducted within the period of annual training cycle of preparing for competition. The characteristics of the main parameters of physical development of athletes are presented in Table 1.

Table 1. Parameters of physical development of the studied group of athletes ($M \pm m$)

Body mass, kg	Body length (upright), cm	Lungs vital capacity, ml	Fat, %
83.9 $\pm$ 7.4	188.3 $\pm$ 3.0	5536.4 $\pm$ 494.2	15.7 $\pm$ 4.1

The data of cardiorespiratory system recorded before and after the training are presented in Table 2 - 5.

Table 2. The data of cardiorespiratory system of the studied group of athletes before and after the training ($M \pm m$)

Parameter	before	after
HR, min ⁻¹	65.7±8.1	93.3±11.7 ^{***}
SBP, mmHg	121.8±6.4	132.5±7.2 ^{**}
DBP, mmHg	82.0±5.2	85.9±6.4
PBP, mmHg	39.8±2.6	45.7±5.6 [*]
RR, min ⁻¹	15.6±4.2	19.3±5.2 [*]
Vtid, l	0.637±0.195	0.684±0.228

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

As shown in the Table 2, significant changes in terms of HR, SBP, DBP, PBP, RR (respiratory rate) and Vtid (tidal volume) occur after training, confirming the well-known data on the activation of the function of cardiorespiratory system caused by exercise. According to many researchers the impact of physical activity on the body of an athlete is determined by the activity of parasympathetic branches of autonomic nervous system after physical exercise, which depends on the starting position of HRV registration. It is shown that orthostatic test should be used for an adequate definition of sympathetic branches of autonomic nervous system after physical exercise.[17,18,19]. The values of heart rate variability (HRV) before and after the training are presented in Table 3, showing likely decrease in the variability in all ranges ($p < 0.05$), which is characterized by expressed ($p < 0.01$) decrease in overall power and increase in low-range and high-range components of HRV. The latter happens due to the increasing influence of the sympathetic part of ANS on heart rate [20,21]. Our studies confirmed the data of other researchers who studied the effect of athletes' regular training loads on ANS activity according to HRV indexes. They found a link between the intensity of training loads and the ratio of the activity of the sympathetic and parasympathetic branches of the ANS. [22,23]

Table 3. Changes of HRV impact on training load

Parameter	before	after
TP, ms ²	4160.3 (1984.7; 9239.7)	879.3 (349.7; 1781.7) ^{**}
VLF, ms ²	566.4 (280.6; 1125.7)	178.3 (83.8; 292.6) [*]
LF, ms ²	1218.0 (482.4; 2986.5)	430.6 (169.4; 877.0) [*]
HF, ms ²	1730.6 (630.9; 3041.5)	173.0 (63.2; 735.1) [*]
LF/HF, ms ² /ms ²	0.81 (0.57; 1.57)	2.11 (1.11; 3.81) [*]

* $p < 0.05$, ** $p < 0.01$

To estimate the results of research of variability of heart rate of athletes using SACR a method of percentile analysis was also used. It is based on the determination of individual assessments of individual features considering relevant limits of percentile ranges designed for qualified athletes (Table 4) [16].

Table 4. Boundaries of percentile distribution of HRV parameters in qualified athletes

Parameter	<5	5-25	25-75	75-95	>95
TP, ms ²	<1017.6	1017.6-2450.3	2450.4-7225.0	7226.1-15700.1	>15700.1
VLF, ms ²	<77.4	77.4-207.4	207.5-812.3	812.4-2152.9	>2152.9
LF, ms ²	<201.6	201.6-552.3	552.4-2116.0	2116.1-7885.4	>7885.4
HF, ms ²	<265.7	265.7-835.2	835.3-3481.0	3481.1-7551.6	>7551.6
LF/HF, ms ² /ms ²	<0.13	0.13-0.37	0.38-1.47	1.48-5.53	>5.53

The results of previous researches make it possible to conclude that in response to training the reaction of a cardiorespiratory system is accompanied by a number of regulatory alterations [24, 25]. Rather informative looks the decrease of autonomic regulatory impacts on HR after training, which can be explained by turning on the regulatory mechanisms with maximum involvement of inotropic function of the heart during exercises, inacting hemodynamic mechanisms ensuring physical activity.

It is obvious that such a mechanism of HR adaptation during exercises is associated with the reduction of influence of all (suprasegmental, sympathetic and parasympathetic) regulatory parts of ANS. At the same time, during a period after exercise activity ANS impact on HR begins to increase, primarily due to the activation of the parasympathetic division. According to our data, 38.7% of athletes reached the optimal level of HF and 32.3% the best level of autonomic balance.

In general, the analysis of individual changes of HRV parameters under the influence of training revealed the following: moderate and expressed decrease in general volume of HRV (TP, ms²) due to larger variety of options: moderate and expressed decrease in VLF (from 15.6% before to 70.9% after training), moderate and expressed decrease in LF (from 15.7% before to 58.1% after training), expressed decrease in HF (from 6.3% before to 58.1% after training) and moderate and expressed increase in ratio LF/HF (from 21.9% before to 58.1% after training). The of the greatest interest in this case are changes of HF-components: 58.1% of athletes were observed to have a decrease to less than 265.7 ms², and some athletes (38.7%) remained within the optimal (835.3-3481.0 ms²) range.

The results prompted us to investigate how the mentioned athletes can be differentiated according to hemodynamic provision of their bodies. 2 groups were formed: the first group (EG₁) consisted of 17, the second (EG₂) of 15 athletes.

The characteristics of the main parameters of physical development of EG₁ and EG₂ are presented in Table 5.

Table 5 The parameters characteristics of physical development of athletes from the studied group

Parameter	EG ₁	EG ₂
Body mass, kg	72.0 (70.0; 76.0)	79.5 (76; 85.5) *
Body length (upright), cm	184 (181; 190)	186 (184; 189)
BMI, kg /m ²	21.4 (20.8; 22.4)	22.4 (22.1; 22.9) *
Shoulder diameter, cm	41 (40; 42)	41.5 (40; 43)
Neck circumference, cm	38 (37; 39)	38 (36.5; 39)
Abdominal circumference, cm	77 (74; 80)	79 (76.5; 84)
Chest circumference, cm	96 (94; 99)	97 (94; 100)
Thorax mobility , cm	8 (7; 9)	9 (7; 10.5)
Mid-arm muscle circumference (relaxed), cm	29 (28; 29)	30 (29.5; 31) *
Mid-arm muscle circumference (tense), cm	33 (32; 34.5)	35 (32.8; 35.5) *
Forearm circumference , cm	28 (27; 28)	28 (26.5; 29)
Hip circumference, cm	52 (48; 56)	54 (50.5; 56.5)
Tibia circumference, cm	36 (34; 37)	38 (37; 39) *
Lungs vital capacity, ml	4900 (4400; 5600)	5200 (4850; 5250)
Fat, %	9.8 (8.1; 13.5)	12.4 (8.9; 17.9)

* - p < 0.05

First of all, analyzing the data presented in Table 5, it should be noted that the differences in the changes of HF-components HRV after training are associated with the physical development of athletes, such as anthropometric and componential elements. This primarily refers to body mass (BM), circumferential size of the arm, leg, the absolute values of which are marked by probable differences, which are characterized by their increase in EG₂ and percentage of fat (Fat, %), the difference of which in EG₁ and EG₂, though not probable, however, suggests an increase in these anthropometric parameters in EG₂ due to fat content. But probably it could be argued that the optimization of changes of HF-components HRV after training within a population range is characteristic for the athletes with more contours size of limbs.

Table. 6 shows comparative parameters of central hemodynamics of EG₁ and EG₂ athletes.

Table 6 Parameters of central hemodynamics of athletes from the studied group

Parameter	EG ₁	EG ₂
EDV, cm ³	92.5 (87.0; 107.6)	116.3 (107.1; 118.8)**
ESV, cm ³	27.1 (22.4; 33.7)	37.2 (33.9; 39.2)**
SV, cm ³	64.9 (61.6; 77.1)	78.7 (72.5; 79.8)*
CO, l	4.4 (4.0; 5.1)	4.8 (4.5; 5.4)*
PVR, dyn×s×cm ⁻⁵	1731.7 (1545.2; 1872.1)	1739.3 (1428.4; 1785.3)
CI, l/m ²	2.32 (2.14; 2.63)	2.45 (2.26; 2.59)

** - $p < 0.01$; * - $p < 0.05$

Figure 1 presents the data on median and procental limits of stroke volume index (SVI ml / m²), the values of which are different for the studied groups of athletes, but not necessarily obvious. The analysis of the types of central hemodynamics revealed that the vast majority of EG₁ athletes are noted by hypokinetic type of blood circulation (88.2%), while the EG₂ athletes' hypokinetic type of blood circulation was observed only in 64% of cases. None of the groups possessed hyperkinetic type of circulation.

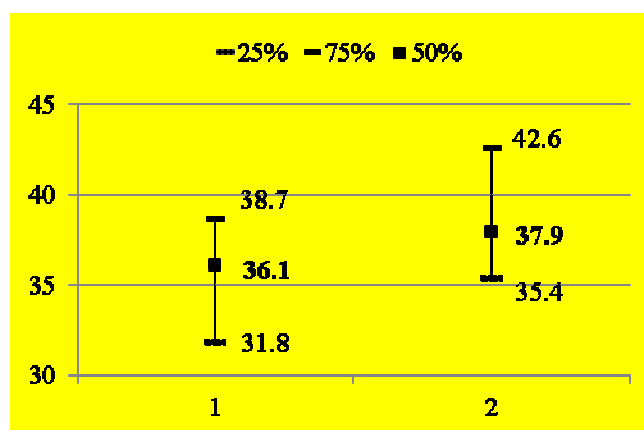


Fig. 1. Median and procental (25% and 75%) values of SVI (ml/m²) for the studied groups of athletes. Where 1 is EG₁ and 2 is EG₂.

The comparative analysis of other indicators of central hemodynamics pointed to the likely differences in some of them. First, it is necessary to pay attention to the indicators of central hemodynamics, which speak of the size of the left ventricle, namely end-diastolic volume (EDV, cm³) and end-systolic volume (ESV, cm³), which is significantly higher in EG₂, than in EG₁ group ($p < 0.01$). A stroke volume (SV, cm³) was more likely ($p < 0.05$) to be observed in EG₂ group.

Table 7 shows the results of calculation of α -factor that characterizes baroreflex sensitivity. It is worth reminding that the values of the last prognose the effectiveness of the regulation of cardiac pump functioning in changing conditions of any type of the activity including training [26,27]. Given the differences in values of BRS_{LF} and BRS_{HF} of the studied group it is possible to suggest that they are related to the size of the circumferential limbs and size of the left ventricle of athletes who are likely to be larger in EG₂ group. According to many researchers the variability of HR contributes significantly to changes in arterial baroreflex during exercise, which is provided by the same stroke volume, and the ability of the central baroreflex to regulate BP is largely dependent on its ability to regulate vascular tone at rest and during physical activity, thus confirming the role of vascular muscle in BP support.[28,29,30]. However, besides the characteristics of physical development and central hemodynamics, we can state that the response to training has clearly defined determinants of baroreflex sensitivity, the value of which at rest predicts reactivity of autonomic nervous system, namely its parasympathetic level in the aftermath of the training that can be used for evaluation of recovery mechanisms of athletes.

Table 7. The parameters of arterial baroreflex sensitivity of athletes from the studied group

Parameter	EG ₁	EG ₂
BRS _{LF}	10.7 (8.7; 17.5)	19.8 (17.3; 22.1)**
BRS _{HF}	12.8 (8.9; 24.9)	25.4 (17.0; 29.7)**#

** - $p < 0.01$ between indicator values in EG₁ and EG₂; # - $p < 0.05$ between the indicator values in EG₂

For further characteristics of features providing hemodynamics of athletes with different variants of HRV response to training an analysis of changes in the cardiovascular system of sportsmen of these groups was carried out in response to a standard exercise in a 20 sit-ups in 30 seconds (Table 8-10), which is widely used in the practice of medical control and allows to characterize the type of response to exercise (Martinet Test). Table 8 presents the data of the surveyed athletes at rest and after the Martinet Test after 1,2 and 3 minutes of recovery. It should be mentioned here that the registration of

parameters of the cardiovascular system of athletes during this test is performed by routine methods: pulsometry for 10 seconds on radial artery with further conversion to 1 min. and measurement of SBP (systolic blood pressure), DBP (diastolic blood pressure) and PBP (pulse blood pressure) using a sphygmomanometer (Korotkov method) to within 2 mmHg.

As shown in the Table 8, in the initial state EG₂ athletes were more likely to reach higher value of SBP 126 (120, 130) mmHg against 118 (110, 120) mmHg in EG₁ group ($p < 0.05$) and PBP 42 (40, 60) mmHg compared to 40 (38; 42) mmHg in EG₁ group ($p < 0.05$) with almost identical values of HR and DBP.

That is, higher values of SBP and PBP at rest in EG₂ group at the background of the same values of HR and DBP can be associated with a higher baroreflex sensitivity and circumferential dimensions of the limbs that characterize the optimal level of influence on heart rate in the HF-range after training.

Table 8. Averaged parameters of heart rate and arterial blood pressure in athletes from the studied groups while conducting the Martinet test

Parameter	EG ₁	EG ₂
HR rest, min ⁻¹	66 (54; 78)	66 (60; 72)
SBP rest, mmHg	118 (110; 120)	126 (120; 130)*
DBP rest, mmHg	80 (70; 82)	80 (76; 82)
PBP rest, mmHg	40 (38; 42)	42 (40; 60)*
HR restitution (0'00''), min ⁻¹	96 (96; 102)	96 (90; 108)
HR restitution (0'50''), min ⁻¹	66 (60; 72)	60 (54; 78)
SBP restitution (1'), mmHg	130 (128; 140)	150 (146; 154)*
DBP restitution (1'), mmHg	70 (60; 76)	66 (60; 70)
PBP restitution (1'), mmHg	70 (50; 80)	90 (80; 92)*
HR restitution (1'00''), min ⁻¹	66 (60; 66)	60 (54; 60)
HR restitution (1'50''), min ⁻¹	60 (54; 66)	60 (54; 60)
SBP restitution (2'), mmHg	130 (120; 140)	140 (136; 150)*
DBP restitution (2'), mmHg	70 (68; 80)	70 (66; 76)
PBP restitution (2'), mmHg	50 (40; 70)	70 (64; 80)*
HR restitution (2'00''), min ⁻¹	60 (54; 66)	60 (54; 60)
HR restitution (2'50''), min ⁻¹	60 (54; 66)	60 (54; 60)
SBP restitution (3'), mmHg	120 (110; 140)	140 (136; 144)*
DBP restitution (3'), mmHg	74 (70; 80)	72 (68; 76)
PBP restitution (3'), mmHg	50 (40; 60)	72 (70; 80)*

* - $p < 0.05$

There were no probable differences in the values of heart rate immediately after exercise and within 3 minutes of recovery. At the same time, changes in blood pressure were characteristic are were related to SBP and PBP, which was reflected in a substantial credible increase in absolute values in response to stress and probably slower recovery within 3 minutes after exercise in EG₂ athletes compared to EG₁ group. That is, from the standpoint of economization of the function of the cardiovascular system the parameters registered in EG₁ group proved to be more favorable.

At the same time, while analyzing the data presented in Table 9, which shows the growth of the parameters of the cardiovascular system in response to a standard load, it should be noted that the only likely differences were observed in terms of QRI ($p < 0.05$).

Table 9. The growth of the performance of the cardiovascular system of athletes from the studied groups in response to load, %

Parameter	EG ₁	EG ₂
growth HR, %	64.1±20.6	61.0±18.0
growth SBP, %	17.6±5.2	15.4±3.2
growth DBP, %	-12.2±9.3	-16.9±5.3
growth PBP, %	72.6±26.7	60.8±16.6
QRI, c.u.	0.83±0.36	1.03±0.30*

* - $p < 0.05$

The latter allows to suggest that the main hemodynamic response to a standard exercise in athletes from EG₁ and EG₂ is defined by baseline of their performance that in terms of SBP and PBP is significantly higher in EG₂ group. However, the percentage of PBP increase was higher than the percentage of HR in athletes from EG₁, while they are the same in group EG₂. To make quantitative analysis of these changes Kushelevskyi and Ziskin offered Quality Reaction Index (QRI), the fluctuations of which are normally within 0.5 – 1.0. At the same time, EG₂ group's average data of this index go beyond the upper limit, which indicates a certain discrepancy in chronotropic and inotropic functions of the heart due to the impact of training in this group.

Table 10 presents data of changes in the cardiovascular system at the end of the recovery after standard load comparing with baseline data.

Table 10. The growth of performance of the cardiovascular system of athletes from studied groups at the 3d minute of recovery comparing with baseline data,%

Parameter	EG ₁	EG ₂
growth HR, %	-2.5±3.6	-3.0±5.1
growth SBP, %	9.4±5.4	6.9±1.3
growth DBP, %	0.3±5.1	-9.9±4.0*
growth PBP, %	25.0±18.2	30.5±7.0

* - p < 0.05

The adequacy of response to standard exercise is determined by gradual recovery of the parameters of cardiovascular system, especially the heart rate, to the third minute of the period of restitution, revealing the level of tolerance of an athlete to physical activity.

According to the data presented in Table 10, heart rate significantly restored to the original level and even lower in both groups of athletes to the end of the third minute after a standard load. At the same time, SBP and especially PBP figures were higher than original. Likely reduce of DBP is noteworthy here comparing to baseline data in EG₂ group unlike EG₁, which according to ordinary data reached almost 10%.

Registered by us differentiation of changes in HF-components HRV of water polo athletes in pre-contest period of annual training cycle (after training to develop speed endurance) revealed that it is determined by certain features of physical development and response of the cardiovascular system to a standard exercise. The latter can be used as prognostic criteria for fitness of athletes during current and phased control in annual training cycle.

First, it is necessary to point out the differences between the athletes of studied groups while undergoing medical control traditionally. The research results of EG₂ athletes comparing with athletes from EG₁ revealed that HF-component HRV optimization at the population level after training is characterized by a greater percentage of fat and significantly larger body size and circumferential arm and leg in comparison with athletes who are noted for an expressed decrease in HF-component HRV. The study of parameters of the cardiovascular system at rest and after a standard load revealed that HF-component HRV optimization after training is associated with significantly higher levels of SBP and PBP in the initial state and within the dynamics of 3 minutes of recovery after standard load and greater decrease in DBP at the end of the period of restitution, which reached almost 10% in comparison with the original level. At the same time, quantitative characterization of response to standard exercise (QRI) suggests that HF-component HRV optimization (EG₂) after training mismatches reactions of chronotropic and inotropic cardiac functioning.

Rather informative were the values that indicate the size of the cavity of the left ventricle (EDV, cm³ and ESV, cm³), which after exercise were significantly higher (p<0.01), significantly higher (p<0.05), were defined as SV (cm³) and cardiac output (CO,l) in the athletes with optimal values of HF (ms²).

4. CONCLUSION

In general, the research revealed that the mentioned features of changes in heart rate variability in the high-frequency range after training have rather accurate determinants in hemodynamic securing an athlete, which in turn can be used to predict and adequately assess the state of the athlete in the recovery period after the competition.

ETHICAL APPROVAL (WHERE EVER APPLICABLE)

All authors hereby declare that all experiments have been examined and approved by the appropriate ethics committee and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

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