The Role of Cholinesterases in Post-Exercise HRV Recovery in University Volleyball Players

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Abstract: Some studies show interest in measuring heart rate variability (HRV) during post-exercise recovery. It is known that the parasympathetic system is relevant during this process, where one of the factors of this modulation is the interaction of acetylcholine and cholinesterases (ChE). However, the behavior of ChE and its relationship during recovery is little known; therefore, the objective of this study was to analyze the behavior of ChE and its relationship with recovery evaluated in HRV indicators in volleyball players. An exercise protocol with long-term and intermittent high-intensity phases was applied in nine volleyball players. HRV measurements were made, and blood samples were drawn to evaluate the ChE before exercise and after 24 and 48 h post-exercise. The results show a modification of the variables after exercises with respect to the baseline values (ChE: 1818.4 ± 588.75 to 2218.78 ± 1101.58; RMSSD: 42.64 ± 12.86 to 17.72 ± 12.55 (p < 0.05); SS: 8.76 ± 1.93 to 21.93 ± 10.05 (p < 0.01); S/PS Ratio: 0.32 ± 0.14 to 3.26 ± 3.28 (p < 0.01)), as well as recovery after 24 and 48 h with respect to postexercise (ChE: 1608.81 ± 546.88 (p < 0.05) and 1454.54 ± 580.45 (p < 0.01); RMSSD: 43.83 ± 24.50 and 46.18 ± 33.22 (p < 0.01); SS: 10.93 ± 5.16 and 11.86 ± 4.32 (p < 0.01); S/PS Ratio: 0.46 ± 0.32 and 0.50 ± 0.28 (p < 0.01)). ChE correlations (p < 0.001) were found with moderate (SS: r = 0.465) and large (RMSSD: r = −0.654; S/PS Ratio: r = 0.666) HRV indexes. In conclusion, ChE modifications are related to changes in HRV showing a very similar behavior in the case of the study subjects.

Keywords: cholinesterases; heart rate variability; autonomic recovery mechanisms; sympathetic–parasympathetic modulation; postexercise recovery

1. Introduction

Training load monitoring has become of vital importance to achieve better exercise adaptations [1,2]. Postexercise recovery plays an important role in this working load monitoring where there is great interest in the use of heart rate variability (HRV), since it is an easily accessible noninvasive tool [3–5] for monitoring the state of the autonomous nervous system (ANS) through its sympathetic and parasympathetic branches [6]. It is also useful for athlete monitoring [7,8], since it allows observing adaptations to training by measuring the fatigue and recovery state [9]. One of the most analyzed indexes of HRV is the root mean square of successive RR interval differences (RMSSD) or its natural logarithm (lnRMSSD) to reduce interindividual variations [3,10]. Recently, Naranjo Orellana et al. [11] published two new HRV indexes, the stress score (SS) and the sympathetic–parasympathetic ratio (S/PS ratio), which provide another viewpoint regarding sympathetic activity and the balance between both branches of the ANS, respectively.
A large number of studies have focused on measuring these variables in postexercise recovery and in determining how the duration and intensity affect them [5,12–14]. However, there is a discrepancy in the use of these results, since, as far as we know, this tool has not been considered for evaluating the internal load [15]. Recovery is a complex interaction between the sympathetic and the parasympathetic system [5,12], where the sympathetic influence is measured by the release of catecholamines and the activation of adrenergic receptors, and the parasympathetic influence is controlled by the release of acetylcholine in the vagus nerve increasing the parasympathetic tone [6]. However, during recovery, parasympathetic activity becomes relevant in returning the organism to a stable state [12,16,17]; thus, afferent vagal stimulation will lead to a reflex excitation of efferent vagal activity that inhibits sympathetic activity [6].

Parasympathetic control depends on the release of acetylcholine from the vagus nerve, which will bind to muscarinic receptors that hyperpolarize the cardiac muscle to decrease the slope of depolarization, resulting in a slowing of the heart rate [18,19]. Efferent parasympathetic control is mediated by cholinergic signaling in the sinoatrial node, which is rich in acetylcholinesterase (AChE), which hydrolyzes acetylcholine after vagal impulses [6,20,21] that will maintain the parasympathetic tone [22,23]. Several studies have analyzed the inhibition of AChE using pyridostigmine bromide to increase the parasympathetic activity, measuring it by HRV [20,24,25].

Since the cholinergic system is involved in the process of ANS modulation, its measurement could provide information on the regulation of the parasympathetic activity. However, clinical measurements of ACh are complicated [26]; therefore, cholinesterase (ChE) measurements could provide this information, as plasma total free ChE has been reported to determine the hydrolysis capacity of ACh [27] and is effective in observing cholinergic events [28]. It seems logical to think that cholinesterases (ChE), divided into the isoenzymes AChE and butyrylcholinesterase (BChE), are the enzymes that hydrolyze ACh descending into muscles after physical exercise to favor parasympathetic activity, thus increase their concentration in the blood.

However, no studies have been found regarding the behavior of ChE in postexercise situations, relating it to HRV recovery. To our knowledge, two studies measure ChE after physical exercise but without associating it with postexercise recovery. One of these [29] assessed whether gender and a physical activity session could modify the AChE and BChE values in people not exposed to pesticides, concluding that physical activity modifies ChE levels after an exercise session. The other [30] analyzed the metabolic responses of traditional clinical markers of the liver; muscle; heart; and bone function (ChE, creatine kinase, lactate dehydrogenase, alkaline phosphatase, etc.) in response to a session of aerobic medium-long distance free running in football players. The authors concluded that these markers provided valuable information on postexercise muscle conditions. However, they mention that ChE should not be used as a marker of the metabolic response, possibly because it was analyzed as a marker of liver damage in this study. We assume at a theoretical level that it could be novel to study the behavior of ChE during recovery and its possible relationship with the ANS regulation process.

We hypothesize that ChE behavior is associated with the process of ANS modulation in postexercise recovery and may be a marker of internal loading. Therefore, the objective of this study was to analyze the influence of ChE behavior on ANS modulation, as assessed across HRV indicators after a combined long-duration and intermittent high-intensity phases exercise in collegiate volleyball players.

2. Materials and Methods
2.1. Headings

This was a quasi-experimental study with an explicit–correlative scope. The study began with a medical examination of the study subjects, measures of body composition, and a stress test, followed by the application of a physical exercise intervention protocol. After the intervention, the subjects had a recovery period of 48 h concentrated in the
installations, controlling rest, the intake of any supplements or drugs that could affect the results, and food. The study duration had a total of 15 days.

2.2. Subjects

A total of nine volunteer volleyball players (21.44 ± 2.07 years; 77.38 ± 6.44 kg body weight; 185.82 ± 10.71 cm height; 25.48 ± 2.50% adipose mass; 48.07 ± 1.86% muscle mass; 18.33 ± 5.16% fat mass; and 49.47 ± 6.36 mL·kg⁻¹·min⁻¹ VO₂max) from the representative team of the Universidad Autónoma de Nuevo León were studied. Sample selection was non-probabilistic, and size was determined using the statistical package G*Power version 3.1.9 (G*Power, Heinrich-Heine, Universität Düsseldorf, Germany) with a probabilistic error of α = 0.05, a statistical power of the probabilistic error of 1 − β = 0.80, and an effect size of d = 1.20.

An informative meeting was held regarding the research objectives, and afterward, all the subjects signed written informed consent for their participation in the study. This study was carried out in accordance with the recommendations of the Ethics Committee of the Universidad Autónoma de Nuevo León (COBICIS-16.09/2012.01GHC), following the ethical standards of all the principles expressed in the Helsinki Declaration [31].

2.3. Procedure

The research was carried out during a training recess of the volleyball players in the winter vacation period. The assessment began with a medical history, physical examination, a blood sample and an HRV measure as a baseline (BASELINE), a body composition analysis (subjects were asked as much as possible to come with the bladder and bowel emptied) by dual X-ray densitometry (DXA (Lunar Prodigy, GE Healthcare, Madison, WI, USA)), kinanthropometry using the complete profile proposed by the International Society for the Advancement of Kinanthropometry (ISAK) [32], and a stress test. These measures were carried out during the first 12 days of the study, as shown in Figure 1.

![Figure 1. Experimental approach.](image)

To determine the VO₂max, an incremental stress test was performed starting at 8 km·h⁻¹ and increasing 0.5 km·h⁻¹ every 30 s until exhaustion with a COSMED Quark PFT ergospirometer (COSMED The Metabolic Company, Rome, Italy) and a TuffTread NF4616HRT treadmill (Tuff Tread, Conroe, TX, USA). VO₂ was determined when a VO₂ concentration plateau was reached; if this plateau was not reached, the value considered was when the respiratory coefficient was greater than 1.15, and the theoretical maximum heart rate was greater than 95%. In this way, the maximum aerobic speed (MAS) was established as the starting speed of the VO₂ max plateau. The methodology proposed by Skinner and McLellan [33] was used to obtain the ventilatory thresholds.

Afterward, a high-intensity and long-duration intermittent exercise protocol was applied to produce elevated physiological stress. Then, blood samples were drawn, and HRV during the immediate recovery period (AFTER) and at 24 (24H) and 48 (48H) hours was measured. The subjects were concentrated in the installations of the university where...
they avoided stimulating food, supplements, or drugs that could alter the results. Food intake (they were given a balanced, proportional, and correct diet) and rest were controlled during the postexercise recovery process.

2.4. Blood Samples

The extraction area was sterilized to obtain the blood sample, and a puncture was made in the median cubital vein with a double-bevel needle, collecting the sample in a 4-mL tube with the anticoagulant EDTA (K2 EDTA, BD Vacutainer, Franklin Lakes, NJ, USA). Afterward, the samples were placed in a Solbat J-40 (SOLBAT S.A. de C.V., Puebla, Mexico) centrifuge at 3000 rpm for 10 min to obtain plasma. Aliquots of plasma were placed in 1.5-mL Eppendorf tubes and stored at −20 °C.

2.5. Exercise Protocol

To obtain a physical demand greater than at a competition and higher physiological stress in volleyball players, a combined exercise protocol was designed based on the Loughborough Intermittent Test [34]. The aim of this protocol was to equal the physical load on athletes to avoid unequal physical loads that commonly occur in team sports during competition, depending on the time of play of the athletes. The VT2 speed percentage reached was used to determine the mean intensity of work for each block.

The protocol consisted of 6 work blocks with 3 min of passive recovery between each. The sequence of the first five blocks was 3 rounds of walking, 3 jumps over a 50-cm hurdle, 1 maximum sprint round, three trotting rounds, and 3 maximum sprint rounds; each round was continuous without recovery with a distance of 20 m and repeating each of the blocks five times. Last, the sixth block consisted of performing a round trotting and a maximum sprint round, repeating these until no more rounds could be performed.

2.6. Cholinesterases

An ELISA in 96-well microplates was used for total and overall concentration of AChE and BChE, following the protocol of the Human Acetylcholinesterase/ACHE ELISA kit (Du- oSet ELISA Development System, R&D Systems, Minneapolis, MN, USA). This was done in duplicate, placing 100 µL of the standard solution and its dilutions for the calibration curve and the subjects’ plasma samples. Afterward, concentration readings were performed with a Bio-Rad iMark Microplate Reader spectrophotometer (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at a wavelength of 450 nm. To calculate the results, a four-parameter logistic (4PL) curve formula was used for logistic regression. For the BASAL level, two nonconsecutive measures were performed. The coefficient of determination of the linear regression of the real and the measured concentration was calculated with an \( R^2 = 0.995 \) and an ICC reliability = 0.998, \( p < 0.001 \). For the baseline measure, the determination coefficient was \( R^2 = 0.878 \) and the ICC reliability = 0.853 (95% CI = 0.731; 0.984); \( p < 0.01 \).

2.7. Heart Rate Variability

The heart signal was recorded using the Polar Team 2 system (Polar Team\(^2\), Polar Electro OY, Kempele, Finland). Ten-minute recordings at rest lying down supine were obtained every day of the week (6:00 a.m.–8:00 a.m.). Data were transferred to the Polar ProTrainer\(^TM\) software version 1.4.5 in its beat-to-beat version to extract the time series (R-R). This temporary series was exported to Kubios software version 2.2 (Kubios HRV Analysis Software, The MathWorks Inc, University of Eastern Finland, Kuopio, Finland) for filtration and analysis, obtaining the RMSSD, SD1, and SD2 parameters. Later, the S:S and S:PS ratio values were obtained using the protocols proposed by Naranjo Orellana et al. [11].

2.8. Statistical Analysis

Statistical analysis was carried out using SPSS statistical software version 25 (IBM Corp., Armonk, NY, USA) with a significance level of \( p < 0.05 \) for all analyses. Descriptive data
are presented as means and standard deviations. A normality analysis was performed with the Shapiro–Wilk test, and Friedman with a Wilcoxon post-hoc tests were used to compare means. Regarding ChE, a reliability analysis was performed using the intraclass correlation coefficient (ICC) and linear regression with a coefficient of determination ($R^2$). Subsequently, the data were normalized using the Z-Score to adjust for a change in intra-subject values in inter-subject samples. To observe the relationship between variables, Pearson’s correlation was used, and for the interpretation of correlation magnitudes ($r$), the following criteria were adopted: $\leq 0.10$, trivial; $>0.10$ to $0.30$, small; $>0.30$ to $0.50$, moderate; $>0.50$ to $0.70$, large; $>0.70$ to $0.90$, very large; $>0.90$, extremely large; and 1.0, perfect [35].

3. Results

The training load was similar for all study subjects with regards to the first five blocks. In relation to block 6, the individual workload varied a little because of the characteristics. Table 1 shows the workload performed by the participants.

Table 1. Training load of the exercise protocol.

| Variable                  | M    | SD  | CV (%) |
|---------------------------|------|-----|--------|
| Blocks 1–5                |      |     |        |
| Total distance (m)        | 7500 | 0   | 0      |
| Mean speed (km·h$^{-1}$)  | 8.7  | 0.8 | 9.1    |
| VT2 speed (km·h$^{-1}$)   | 14.9 | 0.9 | 6.3    |
| VT2 (%)                   | 58.6 | 6.4 | 10.9   |
| Block 6                   |      |     |        |
| Distance (m)              | 546  | 322.2 | 58.9  |
| Mean speed (km·h$^{-1}$)  | 11   | 0.7 | 6.6    |
| VT2 (%)                   | 74   | 2.3 | 3.1    |
| Total exercise protocol   |      |     |        |
| Total time (min)          | 52.2 | 5   | 9.6    |
| Total distance (m)        | 8046.7 | 322.2 | 4     |

VT2: second ventilatory threshold; M: mean; SD: standard deviation; CV: coefficient of variation.

Regarding the descriptive values of ChE, the RMSSD, SS, and the S:PS ratio for the different measures, these are shown in Table 2 with the means and standard deviation.

Table 2. Descriptive analysis of the analyzed variables.

| Variable          | BASELINE | AFTER  | 24H   | 48H   |
|-------------------|----------|--------|-------|-------|
| ChE (pg/mL)       | M        | 1818.41| 2218.78| 1608.81| 1454.54|
|                   | SD       | 588.75 | 1101.58| 546.88 | 580.45 |
| RMSSD (ms)        | M        | 42.64  | 17.72 | 43.83 | 46.18 |
|                   | SD       | 12.86  | 12.55 | 24.50 | 33.22 |
| SS (AU)           | M        | 8.76   | 21.93 | 10.93 | 11.86 |
|                   | SD       | 1.93   | 10.05 | 5.16  | 4.32  |
| S:PS ratio (AU)   | M        | 0.32   | 3.26  | 0.46  | 0.50  |
|                   | SD       | 0.14   | 3.28  | 0.32  | 0.28  |

ChE: cholinesterase; RMSSD: root mean square of successive RR interval differences; SS: stress score; S:PS ratio: sympathetic–parasympathetic ratio; M: mean; SD: standard deviation.

Figure 2 shows the behavior of the analyzed variables, which shows that ChE, SS, and the S:PS ratio had a greater elevation in the AFTER measure compared to the BASELINE measure, with a significant change of the SS, as well as the S:PS ratio ($p < 0.01$). The RMSSD has a significant descent ($p < 0.05$) in the AFTER measure compared to the BASELINE measure. All significantly returns to baseline levels at 24H and 48H ($p < 0.01$), except for ChE, which decreases significantly more than its baseline level ($p < 0.05$ and $p < 0.01$) compared to AFTER; in the 48H measure, it continues to descend with a significant change ($p < 0.05$) compared to 24H.
The relationship of the behavior between variables during the measures is shown in Table 3, where statistically significant mean and high correlations between all the variables can be seen. The correlations of ChE with the HRV indexes are presented graphically in Figure 3 by linear regressions to observe the trend line adjustment and the coefficient of determination ($R^2$).

**Table 3. Correlation between the variables ChE, RMSSD, SS, and the S:PS ratio.**

|          | ChE       | RMSSD    | SS       |
|----------|-----------|----------|----------|
| RMSSD    | Pearson’s correlation | $-0.654^{**}$ |          |
| SS       | Pearson’s correlation | $0.465^{**}$ | $-0.879^{**}$ |
| S:PS ratio | Pearson’s correlation | $0.666^{**}$ | $-0.926^{**}$ | $0.942^{**}$ |

**Two-tailed bilateral significance at the level $p < 0.001$. ChE: cholinesterase; RMSSD: root mean square of successive RR interval differences; SS: stress score; S:PS ratio: sympathetic-parasympathetic ratio.**

**Figure 3.** Correlation $R^2$ of the ChE with the root mean square of successive RR interval differences (A), the stress score (B), and the sympathetic/parasympathetic ratio (C).
4. Discussion

The main contribution of our study is that ChE shows a behavior during postexercise recovery that is similar to the RMSSD indexes, SS, and S:PS ratio of HRV; therefore, we can consider these internal load markers.

It has been described in the literature that during a postexercise recovery, the interaction of the parasympathetic and sympathetic systems is complex [5,12] and mediated mainly by parasympathetic reactivation [36,37]. This includes multiple regulations such as the descent of circulating catecholamines, blood pressure, baroreflexes, and metaboloreflexes that cause a drop in sympathetic stimulation [4,5,17] due to an efferent reflex effect of vagal stimulation [6].

This effect is regulated by cholinergic signaling in the sinoatrial node, and in a certain way, ChE are mediators of the vagal impulse by hydrolyzing ACh [6,20,21]; thus, an increase of ChE in blood indirectly reflects an increase in the rate of ACh hydrolysis leading to ACh descent [27,38,39]. Our results support this idea since, in postexercise measures, the increase of ChE widely correlated with an increase in sympathetic activity (SS and S:PS ratio) and a decrease in RMSSD (Table 3). Afterward, in the 24H and 48H measures, ChE normalized, and RMSSD returned to her initial values, as well as the SS and the S:PS ratio. These changes may be because AChE facilitates the signaling of the onset and termination of the cardioinhibitory effect by hydrolyzing ACh in the synaptic cleft in a very short time (<1 s) [4,40]. Subsequently, the parasympathetic nerve will be stimulated in the phasic mode, where released acetylcholine will hyperpolarize the sinoatrial node by binding to its muscarinic receptors decreasing the depolarization slope and thereby delaying the next sinoatrial node impulse [18,19,41] and, thus, favoring postexercise HRV recovery.

This seems to indicate that these variables evaluate the internal load regardless of the type of exercise performed since, although some authors relate the recovery with duration [4,42] or with intensity [5,43], in our results, it can be seen that the workload was very similar for all subjects (Table 1), demonstrating that the changes in response show the individual assimilation of that load. Therefore, the changes in these variables would fit into the concept of internal load. The indexes, RMSSD, SS, and S:PS ratio have been used jointly in various studies to see the behavior of the sympathetic and parasympathetic systems, both in training control and in competition, concluding that these indicators are valid and reliable for training and competence monitoring [13,44–46].

On the other hand, cholinesterase values (Table 2) are within the range of acceptable clinical values for the technique used (1278 ± 338 pg/mL), so the changes observed in this variable are not at any time abnormal or pathological values. Thus, we observed that the postexercise ChE behavior coincides with the increase reported in the study by Zimmer et al. [29]. However, this does not seem to be the case with that reported by Chamera et al. [30], as the behavior of ChE in women is very similar to our results but not so in men. This could be because the impact of the session was not sufficiently demanding to provoke high rates of ACh hydrolysis. The values of RMSSD, SS, and the S:PS ratio coincide with those presented by other authors [13,44,47], and they would be within the 25–50 percentile of the reference tables reported by Corrales et al. [47]. The behavior of HRV indices following such an exercise is similar to what has already been well-documented in the literature [13,48], where it has been reported that HRV is a reliable indicator to measure fatigue and postexercise recovery with acceptable relations [3–5,8,9,49], establishing the average values for a good state of the athlete (recovered) of RMSSD above 30–40 ms, SS below 10AU and S:PS ratio below 0.5AU [11,44,50]. In addition, acceptable relationships have been shown with other classical markers of recovery (creatine kinase, testosterone/cortisol ratio, immune/inflammatory markers, and training load) [9,13,51–53].

To support the idea of the role of ChE during recovery, in our results, the correlation of ChE with HRV indexes was observed. This could indicate the modulation effect of the pacemaker potential of the sinoatrial node by parasympathetic innervations mediated by ACh and ChE [6,12,21,36,37,54,55]. This may be explained by the fact that, when central command and mechanoreceptor feedback cease, the arterial baroreflex is reset to a
lower level, increasing the parasympathetic activity and rendering it phasic \cite{36,37,40,56,57}. Since this parasympathetic activity is controlled by the release of ACh from the efferent vagal nerve discharge, its modulation will largely depend on the ChE activity \cite{6,20,21}, supporting this idea by the association found with HRV indices, since as mentioned above, plasma ChE could provide information on cholinergic events \cite{27,28}.

4.1. Limitations

The main limitations of this study are the sample size, which has been set at this number to prioritize the homogeneity offered by being a team contemplating a statistical analysis to obtain the ideal sample size and not having the means that allow us to simultaneously contrast what happens in synaptic transmission. Nevertheless, the relationships found between the variables allow us to reasonably assume the conclusions found and to set a starting point for new studies in this direction.

4.2. Practical Applications

Our results describe the behavior of ChE and how it is associated with the recovery of parasympathetic activity postexercise, which could be of useful application in understanding—more specifically, the behavior of HRV indices following a training load similar to that of this study.

5. Conclusions

The modifications observed in ChE appear to influence the changes in sympathetic and parasympathetic activity, since they show an acceptable relationship with HRV indices, which could improve the physiological interpretation of the recovery of parasympathetic activity after a combined long-duration and intermittent high-intensity phases exercise. These findings might have relevance for specialists in the control of training areas for future research, since these results suggest that the ChE and HRV levels might be considered internal load markers.

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