Reference Change Values for Immature Red Blood Cell Parameters in Under-20 Soccer Players

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Abstract

Introduction: Immature red blood cell parameters compose of the anti-doping programs of several sports, including soccer. The biological variability of reticulocytes is not well defined in young athletes. Our aim was to calculate biological variation and reference change values (RCV) for immature red blood cell parameters in young soccer players.

Methods: Samples from 19 male soccer players (mean age, 18 ± 1 years) were collected before the start of training (C0) and after two (C1) and four weeks of training. Blood samples were collected in tubes with K₃-EDTA Vacuette® (Greiner Bio-One). Red blood cell parameters were analyzed on Sysmex XE-5000®. The e-Check Sysmex® 2 levels were used to obtain the coefficient of analytical variation (CV_A). Homogeneity of variance was verified using the Cochran test. The within-subject (CV_I) and between-subject biological variation (CV_G) was calculated according to the mean and standard deviation from the athletes' results. ANOVA with repeated measures was used to compare the means at the significance level of $p < 0.05$. RCV_{95%} was calculated using the Fraser's formula: $RCV = 2^{1/2} \times 1.96 \times (CV_A^2 + CV_I^2)^{1/2}$. Graph Pad Prism 6.0 and Matlab 7.0 were used to perform statistical analyzes.

Results: Mean corpuscular volume, RDW, and reticulocyte hemoglobin content (Ret-He) had significant increases during the training period. The Ret-He were higher in C1 (32.4 ± 1.1 pg) and C2 (32.3 ± 1.3 pg) compared to C0 (31.9 ± 1.2 pg) ($p < 0.05$). It was not possible to calculate RCV for Ret-He because of heterogeneous variances. The RCV of reticulocytes (29.1%) and IRF (48.8%) were higher than Hgb (5.5%) and Hct (6.7%).

Conclusion: Reticulocytes are highly variable in different athletes' and the RCV obtained from young soccer players would contribute to monitor training adaptation and to diagnose sports anemia. The Ret-He behavior in our study suggests that this parameter can detect an early increase of iron for hemoglobin synthesis in response to training, this makes it an interesting biomarker to assess anemia and suspicion of doping by rhEPO.

Introduction

The longitudinal monitoring of blood cell parameters are of increasing importance in the sports medicine to prevent training-related injury ¹, to indicate changes in immune function ², pathologies ³ or of anti-doping purposes ⁴. With the emerging concept of the athlete biological passport (ABP) introduced by the World Anti-Doping Agency (WADA) in 2008, several sports federations adopt the hemogram for disciplinary purposes⁵. The hematological module of ABP can be assessed to identify possible blood doping methods, such as the enhancement of oxygen transport by recombinant erythropoietin (rHuEpo) abuse, erythropoiesis-stimulating agents, blood transfusion and exogenous testosterone. ^{6,7} However, some of ABP components such as hemoglobin concentration ([Hb]) and hematocrit (Ht) could be influenced by plasma volume changes ⁸ and training.^{4,9} To increase the sensitivity of ABP others

biomarkers such as reticulocytes % (Ret %), immature reticulocyte fractions (IRF), platelets count, leukocytes count and OFF-hr score (an index that combines [hb] and Ret %), are analyzed longitudinally taking account the within-subject biological variation through a Bayesian approach^{6,10}.

Automated hematology analyzers can quantify reticulocytes using a wide variety of reagents for reticulocyte RNA, improving precision and accuracy compared to manual methods. Besides, it performs reliable measurements of mRNA content and cellular indices such as reticulocytes volume, hemoglobin content and IRF.¹¹ These immature red blood cell (RBC) parameters can be useful for monitoring positive adaptations to training or diagnosing sports anemia because are not affected by training-induced plasma volume changes.⁹ Immature RBC parameters were successfully established to monitor low iron levels in physically active individuals.¹² Diaz et al showed that reticulocytes are stable in triathletes over the season suggesting that high volume of training does not chronically affect biological variability of this parameter.¹³ However, Banfi & Del Fabbro observed modifications of reticulocytes during training and competition season in different sports disciplines (e.g. soccer, ski, rugby, and cyclism).¹⁴ Likewise, different behavior between sports disciplines, reticulocytes depend on physical fitness (professional or recreational athlete and sedentary people).¹⁵ Red blood cell parameters shown high inter-individuality compared to intra-individuality and the reference change values (RCV) seems to be a better alternative than population-based reference intervals to monitor athletes.⁵

Professional athletes are frequently submitted to blood tests during training and competition season¹. Despite this, young soccer players are not being monitored during training or competition season as professional athletes. The biological variability of immature RBC parameters has not been well defined in young soccer players yet. We aimed to calculate the biological variation and RCV for immature RBC parameters in under-20 soccer players and verify the training effects on reticulocytes parameters.

Materials And Methods

Participants

The study included nineteen male soccer players, age (18 ± 1 years old), height (1.81 ± 0.1 m), weight (74.6 ± 8.7 kg) and body mass index (22.8 ± 1.9 kg/m²). All participants were under-20 soccer players in the preparation for a national competition. The participants gave their written formal consent for participation in the research. The participants filled out a questionnaire about the use of medication and those who were injured or using medications in the last three days were not included in the sample group. The athletes were evaluated before the start (C0), after two (C1) and four weeks (C2) of preparatory training for national under-20 soccer competition. The training period included about 250 minutes per week of physical, technical and tactical activities. This study was approved by the Ethics Committee for Research with Humans (CAAE: 47669515.5.0000.5632). All study procedures were following the Helsinki Declaration.

Blood Sampling and Analysis

All blood samples were collected under standardized conditions after two days of rest to avoid the acute effects of exercise as previously reported¹⁶. Were collected 2.0 mL of total venous blood in vacuum tubes containing EDTA/K3 (Vacuette®, Greiner Bio-one, Brazil) to determine hematological parameters. The blood samples were collected in the morning after 12 hours of fasting, with the subjects in a seated position. All samples were then transported to the laboratory at 4 °C and analyzed within 60 min after the blood collection. The hematological parameters were obtained by a Sysmex XE–5000® automated analyzer that uses a polymethine dye specific for RNA/DNA for reticulocyte enumeration, degree of immaturity and determination of hemoglobin content. The following parameters were analyzed: red blood cell count (RBC), blood-hemoglobin concentration ([Hb]), hematocrit (Ht), mean corpuscular volume (MCV), erythrocyte distribution width (RDW), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), reticulocyte count (Ret), immature reticulocyte fraction (IRF), and reticulocyte hemoglobin content (Ret-He). The e-Check Sysmex 3 levels were used as internal quality control and were performed in parallel with the tests. The mean and standard deviation derived from control samples were used to calculate the coefficient of analytical variation (CV_A). Desirable analytical performance was assumed if the imprecision was such that $CV_A < 0.5 \times CV_I$.¹⁷

Statistical analysis

The Matlab 7.0 software and GraphPad Prism 6.0 were used to perform statistical analyses. Data were tested for Gaussian distribution with the Kolmogorov-Smirnov test. ANOVA with repeated measures was used to compare the means at the significance level of $p < 0.05$. Homogeneity of variance was verified using the Cochran test applying a Matlab function proposed by Trujillo-Ortiz and Hernandez-Walls¹⁸. The within-subject biological variation (CV_I) was estimated using the mean of the individual's results across 3 samples and standard deviation. The between-subject biological variation (CV_B) was calculated according to the mean and standard deviation between the athletes' results. As the calculated average within-subject biological variation includes an analytical component we applied Fraser's Formula¹⁹ to remove the CV_A from CV_I . $RCV_{95\%}$ was calculated using the formula: $2.77 \times (CVA^2 + CVI^2)^{1/2}$ as previously described²⁰. The Factor 2.77 is equal to 2 times (bidirectional change) the z score ($1.96 = 95\%$)^{19,20}.

Results

Table 1 shows the mean and 5th–95th percentile for red blood cell count before start training (C0) and after two (C1) and four (C2) weeks of training from under–20 years old soccer players. MCV, RDW, and Ret-He had significant increases during the training period. MCH and MCHC showed decreased values.

Table 1

Red blood cell count before and after the training period from under-20 soccer players

	C0	C1	C2	<i>p</i>
RBC ($10^{12}/L$)	5.19 (4.8 - 5.9)	5.16 (4.0 - 5.8)	5.26 (4.7 - 6.0)	0.109
Ht (%)	45.3 (41.7 - 49.7)	46.2 (42.2 - 49.6)	46.5 (41.1 - 50.6)	0.275
[Hb] (g/dL)	15.0 (13.2 - 16.9)	15.0 (13.4 - 16.4)	15.1 (13.0 - 16.7)	0.725
MCV (fL)	87.3 (82 - 93)	88.9 (84 - 94) *	88.5 (84 - 94) *	<0.001
MCH (pg)	29 (27 - 31)	29 (27 - 30)	28 (26 - 30) *	0.001
MCHC (g/L)	33.1 (31.7 - 34.2)	32.4 (31.1 - 33.4) *	32.3 (30.9 - 33.6) *	<0.001
RDW (%)	13.2 (12.0 - 14.1)	13.2 (11.9 - 14.2)	13.5 (12.1 - 14.7) *	<0.001
Ret (%)	1.06 (0.63 - 1.59)	1.10 (0.55 - 1.57)	1.11 (0.74 - 1.58)	0.472
Ret ($10^9/L$)	55.5 (30.8 - 85.9)	57.6 (28.1 - 86.1)	58.7 (38.6 - 86.7)	0.445
IRF (%)	7.5 (3.8 - 14.3)	7.1 (2.3 - 12.3)	7.5 (2.9 - 14.0)	0.625
Ret-He (pg)	31.9 (29.4 - 34.4)	32.4 (30.5 - 34.3) *	32.4 (29.1 - 34.2) *	0.024
RBC (red blood cell count); Ht (hematocrit); [Hb] (hemoglobin concentration); MCV (mean corpuscular volume); MCH (mean corpuscular hemoglobin); MCHC (mean corpuscular hemoglobin concentration); RDW (erythrocyte distribution width); Reticulocyte count (Ret); IRF (immature reticulocyte fraction); Ret-He (reticulocyte hemoglobin content).				
Data are expressed as means (5 th - 95 th percentile). * compared to C0.				

Table 2 shows the components of biological variation (CV_I and CV_B), CV_A , and $RCV_{95\%}$ obtained from young soccer players after four weeks of the training period. It was not possible to estimate the biological variation components for MCV ($CV_A = 0.8\%$), MCH ($CV_A = 0.9\%$), MCHC ($CV_A = 1.3\%$), RDW ($CV_A = 1.2\%$), and Ret-He ($CV_A = 0.8$) because the sample variances were heterogeneous. The calculated CV_A of all the parameters in Table 2 was $< 0.5 \times CV_I$, except for IRF ($< 0.75 \times CV_I$).¹⁷

Table 2

Biological variation and RCV_{95%} for red blood cell parameters in under-20 soccer players.

	CV _A (%)	CV _I (%)	CV _B (%)	II	RCV _{95%} (%)
RBC	1.0	2.2	5.5	0.4	6.1
Ht	1.2	2.4	5.1	0.4	6.7
[Hb]	0.9	2.0	6.1	0.3	5.5
Ret	3.7	10.5	27.3	0.4	29.1
IRF	11.2	17.6	38.1	0.4	48.8
RCV (Reference change values); RBC (red blood cell count); Ht (hematocrit); [Hb] (hemoglobin concentration); Ret (reticulocyte count); IRF (immature reticulocyte fraction). CV _A (coefficient of analytical variation); CV _I (within-subject biological variation); CV _B (between-subject biological variation).					

Figure 1 shows the mean, minimum and maximum values for reticulocyte count for each athlete during the study. Reticulocyte count showed higher between-subject biological variation (CV_B = 27.3%) compared to within-biological variation (CV_I = 10.5%) (Table 2).

Figure 2 shows the mean, minimum and maximum values for Ret-He (pg) for each athlete during the study. Two athletes (10,5%) had Ret-He below the cut-off value for iron deficiency (Ret-He = 30.6 pg) ²¹ in the first analysis (C0). However, during the study period all these soccer players had no anemia.

Discussion

Brazil has been the most active country in the international football transfer market since FIFA started collecting transfer information from member associations worldwide in 2010. Brazilians soccer players are transferring out of Brazil on average younger, almost one-third of all transactions were under 18 years old²². However, few studies describe immature red blood cell parameters in these athletes during training periods ¹⁴.

Reticulocytes are immature red blood cells that remain 1–2 days in the bloodstream and provide a good index of hemoglobin in newly produced red blood cells in response to training²¹. In our study, we observed significant increases in Ret-He induced by training (Table 1). To our knowledge, is the first study that shown this behavior in young soccer athletes. Ret-He is used successfully in the diagnostic of functional iron deficiency and iron deficiency anemia in non-exercised individuals.^{21,23} Ret-He represents a direct measurement of iron for hemoglobin synthesis in the reticulocyte and has no limitation in contrast to biochemical iron status parameters para such as ferritin and transferrin that are strongly

influenced by inflammation.³ Our Ret-He values were increased after the training period with no significant changes in the reticulocytes count. This behavior showing early response for this parameter compared to reticulocytes as previously observed after 48 hours after iron therapy in deficiency treated patients.²⁴

Soccer is characterized by high neuromuscular demands with sprints, changes in direction, and jumps. Young soccer athletes play one or two matches per week. Thus, elite soccer training programs are planned to prepare the players to repeat these high efforts several times a week during a whole season²⁵. Important hormones that stimulate the erythropoiesis, such as erythropoietin, testosterone, and growth hormone have shown an increase as a training adaptation.^{26,27} We observed slight differences in traditional hematological indices MCV, MCH, MCHC and RDW (Table 1). However, the mean differences were lower than the biological variation for physically active individuals.²⁰ Lippi et al. observed similar behavior for MCV and RDW after moderate endurance exercise and suggested that these changes could be potential implications on ABP.²⁸

Adequate nutritional status during training periods is a fundamental requirement for positive adaptations and optimal physical fitness in young athletes.³⁶ For endurance modalities, oxygen transportation is crucial to achieving better performance. Hemoglobin is the oxygen carrier to the muscles and the iron is the central component in heme proteins. Hemoglobin and iron status were positively associated with cardiorespiratory and muscular fitness in young athletes.³⁶ During our study period, the hemoglobin concentration remains stable with no significant change. Plasma volume adaptation and the short training period interval of our athletes may explain this behavior.⁸

The RCV observed for traditional RBC parameters (Table 2) were similar when compared to physically active individuals.²⁰ As previously reported in different sports by Banfi et al. [Hb], Ht e RBC have almost identical variation, in contrast to reticulocytes parameters that present high variation.²⁹ Besides, at the end of competition season reticulocytes can decrease between 5 and 21% without alteration in [Hb].¹⁴ It is important to state that our athletes were at the beginning of the season and most of the hematological changes are observed in the heaviest training periods.^{14,29}

Reticulocytes evaluation in soccer players is important because of its usefulness in detecting sports anemia. During some stages of endurance training, plasma volume changes promote hemodilution, instead of hemoglobin concentration could be falsely decreased. Furthermore, athletes could experience exercise-induced hemolysis due to mechanical trauma or oxidative stress. Our reticulocytes biological variation data (Table 2 and Figure 1) showed a low within and high between-subject biological variation. This behavior suggests the RCV than population-based reference intervals to better interpret the athlete's results.³⁰ Endurance athletes have similar results for reticulocytes between-subject variation ($CV_B = 28\%$), but higher values for $CV_I = 21,1\%$ and $RCV_{95\%} = 42,8\%$ than young soccer players.⁵ In a large professional soccer player population, Malcovati et al. calculated a slightly higher reticulocyte within-subject coefficient of variation ($CVI = 16,3\%$).³¹ These differences may be explained by the effect of age, ethnicity,

sports discipline, training phases, and analytical variation.^{5,31,32} Also, intraindividual biological variation for reticulocytes is higher in soccer players compared to inactive subjects.^{33,34}

Immature reticulocytes are cells with high RNA content which results from the increased rate of bone marrow red cell production.¹⁴ In athletes, high IRF values can be seen in hemolysis, altitude training, and blood manipulation with erythropoietic stimulants.³⁵ The biological variation for IRF in our soccer players was slightly higher than observed in the non-athletes study population, repeating the same pattern for reticulocytes and RBC parameters.³⁴

Conclusion

Reticulocytes are highly variable in different athletes' and the RCV obtained from young soccer players would contribute to monitor training adaptation and to diagnose sports anemia. The Ret-He behavior in our study suggests that this parameter can detect an early increase of iron for hemoglobin synthesis in response to training, this makes it an interesting biomarker to assess anemia and suspicion of doping by rhEPO.

Declarations

Competing interests:

The authors declare no competing interests.

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Figures

Figure 1

Mean, minimum and maximum values for each athlete at C0, C1, and C2 for reticulocytes count.

Figure 2

Mean, minimum and maximum values for each athlete at C0 to C2 for Reticulocytes hemoglobin content (Ret-He)