Reticulocyte counts in sports medicine

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Abstract

Reticulocytes are juvenile red blood cells (RBCs) containing remnant ribonucleic acid (RNA). Their percentage in the peripheral blood (PB) is a useful indication of erythropoiesis in the bone marrow. In the context of sport, the reticulocyte count and other complete blood count (CBC) parameters can provide useful information about the potential for athlete performance for trainers and sports medicine clinicians. In order to obtain reliable measurements, pre-analytical variables have to be controlled, including the timing of blood collection and the standardisation of phlebotomy procedures. Although the manual reticulocyte count with supravital staining is recommended as the reference method, the absolute and percentage reticulocytes in whole blood are today better analysed using modern haematology cell counters. The comparability of the results from the manual and automated methods remains in doubt mostly due to the lack of suitable calibration and control bloods for both methods.

Mild haemolysis and increased oxygen demand are associated with exercise, especially during endurance training. The production of erythropoietin is enhanced in this setting inducing erythropoiesis resulting in an elevated PB reticulocyte count. Athlete training at high altitudes produces a physiological PB hypoxic effect in an attempt to maximise oxygen carrying capacity and performance. Studies into the reticulocyte response in athletes show that minimal intensity and duration of training will initiate the erythropoietic response to increase PB reticulocyte numbers. Efforts to raise athlete performance by increasing the number of circulating RBCs have led to the banning of autologous and/or homologous blood transfusions and the use of recombinant human erythropoietin (rHuEpo). The illegal use of these methods to boost performance is a major focus for some athletes and a major challenge for anti-doping agencies trying to keep sport clean from the use of performance enhancing drugs.

Introduction

Immature red cells with remnants of RNA are termed reticulocytes and they are present in both the peripheral blood and the bone marrow (1). As they mature to become functionally normal erythrocytes, the haemoglobin content of the reticulocyte increases and the size of the cell decreases (1). The concentration of reticulocytes in the PB is estimated from a PB specimen with the reticulocyte count a test frequently performed in the haematology laboratory. Most reticulocyte testing in the lab is performed on patient samples, providing useful information about the physiologic BM response to disease and response to medication and other treatments. For some sports trainers and sports medicine clinicians, it is becoming more popular to monitor athlete performance against the effective rate of RBC output from the bone marrow (2). Moreover, during competition seasons, the regular blood sampling of athletes to establish the peripheral blood reticulocyte count now plays an important role in the policing of the illegal use of blood doping in sport (2, 3).

Key words: reticulocyte, recombinant human erythropoietin, blood doping, training, sports

Pre-analytical variables

The control of pre-analytical and analytical variables is crucial for gaining accurate reticulocyte measurements if the reticulocyte count is to be used to detect those athletes using rHuEpo to boost performance. To eliminate variations in the reticulocyte count a number of pre-analytical variables must be standardised. Studies have shown that prolonged tourniquet times during blood collection can produce false haemoglobin (Hb) and haematocrit (Hct) results due to altered cellular fluid distribution. While there is no real evidence that this has an influence on the reticulocyte count it is recommended by doping agencies that a standardised venepuncture procedure for sample collections be followed at all times (2). As with other biological substances, the reticulocyte count shows diurnal variation peaking at around 1:00am with a corresponding rise in the erythropoietin (EPO) concentration (2, 5). Blood collections for reticulocyte counts used to monitor human athletic performance need to be performed at a similar time interval to minimise intra-personal variability. Adherence to a standardised collection time for blood sampling has become an issue for agencies monitoring athletes following intercontinental flights (2).

Generally, reticulocyte numbers in whole blood remain stable for up to 24 hours after collection, if samples are refrigerated. Results can become falsely low if analysis is further delayed as reticulocytes mature in the blood sample prior to testing (1, 2). It is therefore important to ensure the optimal transportation and storage conditions for specimens being tested for reticulocyte numbers in order to show genuine stimulation of the bone marrow in athletes using rHuEpo (2).

Analytical variation

The reference range of the reticulocyte count for the general population is 0.5-2.5%, which is similar to the range expected for athletes (2, 4). It is suggested that a reticulocyte count of less than 0.4% or greater than 2.6% in athletes may be considered abnormal (2). Differences among ethnicities have been reported for some of the reticulocyte related parameters, such as increased values for the reticulocyte haematocrit (Retht) in some African athletes (2).

The reference staining method for the manual reticulocyte count uses a dye such as new methylene blue and supravital staining (2). Standard microscopic examination of blood films classifies reticulocytes as a red cell containing at least two blue dots or strands of filamentous reticulum (1, 2). The subjective, time-consuming and imprecise nature of the manual method has given way to use of analysers in today's laboratories (2, 4). Cell analysers use either supravital staining or a fluorophore technique to measure emitted fluorescence from stained reticulocytes (2, 3). Besides the percentage and absolute number values for the reticulocyte count, the volume (MCVr), immature reticulocyte fraction (IRF) and haemoglobin content of reticulocytes (Chr) can also be provided by some analysers. These can be useful parameters to establish recent reticulocyte stimulation of reticulocytes in response to rHuEpo usage contrasting with results seen in normal physiologic RBC production (2, 4). Research found that the MCVr and Chr values are elevated in athletes either using or having used rHuEpo within the previous three weeks (2).

A number of studies have been done to evaluate the consistency of reticulocyte results measured by various automated haematology analysers. Satisfactory agreement of measurement have been established for some analysers (2, 3). The major problem with testing is the consistency of the reticulocyte count across all
automated platforms (2,3). The lack of a reliable calibrator means the result from one analyser may not be comparable with the results obtained from the same blood on another analyser. Even comparisons of results obtained from the same series of instruments in different laboratories may produce variable results (2). Ashenden et al (2004) introduced a concept of analyser-specific bias using a samplator protocol. This integrated the mean values of a large sample measured at sea level using two analysers and they calculated the bias between the two analysers of interest allowing for the final reticulocyte count to be compensated (3). It is therefore important to factor in analyser variables when using reticulocyte analysis for athletes during training and for anti-doping purposes (2). The variability of analyser estimations of the reticulocyte count in this setting has led to the suggestion that it might be more relevant to compare an athlete's reticulocytes against his/her baseline results. These could be documented in the form of a so-called haematological passport, rather than using a generalised reference range for all athletes (2).

**Effects of training**

Mechanical damage and oxidative stress on circulating red cells are listed as the main causes of RBC destruction during active training, particularly in endurance running (6). The level of haemolysis can be raised in plasma and can be measured with a consequential loss of plasma haptoglobin (2,4,6,7). Increased red cell turnover combined with an expanded plasma volume in athletes can lead to reduced oxygen tension in the kidneys. These events enhance the expression of EPO by hypoxia-inducible factor 1 (HIF-1), with a rise in the reticulocyte count and increased plasma reticulocyte-related iron fraction (IRF) (5-8). Additionally, depleted iron stores that can occur due to excessive loss or insufficient intake over time, can lead to or contribute to existing anaemia, especially in female athletes (2,6,7).

Studies have shown that the percentage of PB reticulocytes can be decreased in athletes and the IRF may show a slightly increase or remain stable during a competitive season. There was no significant correlation between the reticulocyte values across various sports disciplines (2,4). For many athletes the variations observed are still within the physiologically normal reference range (2,4).

The theory of improved performance during training at high altitude or the so-called living high – training low protocol is to trigger the hypoxic response in the body. The supposed improved athletic performance associated with this relies upon an increased RBC mass and adjusted oxygen perfusion to the tissues at altitude. Following a return to a lower altitude the increased RBC mass improves oxygen intake at normal atmospheric pressure leading to better performance among athletes (2,5,8). Julian et al researched the effects of simulated hypoxia on the red cell population. In their experiments the research subjects inhaled intermittently hypoxic and normally pressurised air (5). They found no significant correlation between changes in the haematological values and individual athlete performance (5). In other studies the lack of a control population meant it was not clear whether observed performance improvements in athletes were induced by hypoxia or simply by concentrated training during the studies (2,5). Today it is thought that it takes a minimum altitude of 2100-2500m with a certain intensity and duration of training to initiate a measurable reticulocyte response (2,5,8).

**Blood doping**

Blood doping among athletes is undertaken to illegally and exogenously increase Hb and Hct values to elevate the oxygen carrying capacity of the blood to increase the oxygen supply to muscle tissue and improved physical performance in sport (7). The earliest form of doping involved the transfusion of autologous and/or homologous blood prior to sports events (2,7). These activities exposed athletes to transfusion reactions and transfusion-related infections following unsupervised blood transfusions (7). The autologous transfusion process firstly involves the removal of a large volume of blood inducing an increase of effective erythropoiesis and an elevated reticulocyte count (2). Following the re-infusion of the athletes' autologous blood prior to competition, EPO levels are suppressed and low reticulocyte counts and low levels of soluble transferrin receptors are found in the blood (2,5). An increase of Hb by more than 7.5% in an athlete over a short time interval could be indicative of a recent transfusion and is usually followed up by the anti-doping agencies (2).

**Recombinant human erythropoietin (rHuEpo)**

Erythropoietin is a hormone that regulates bone marrow erythropoiesis. The release of synthetic rHuEpo within the last 10 years has led to its misuse in sport. Recombinant HuEpo has similar properties to natural EPO and has wide applications in medicine for the treatment of patients with renal failure and anaemia caused by chronic disease (1,9). Exogenous or pharmaceutical EPO is being used illegally by some athletes to improve performance in competitive sport in endurance events (2,3,7). A number of health risks are associated with the use of rHuEpo in sport depending upon the dose of the drug. With high dosage there is a marked increase of blood viscosity and polycythaemia including thrombocytosis. Increased blood viscosity can lead to heart failure and increased platelet numbers coupled with increased blood viscosity can expose athletes to a higher risk of the life-threatening venous thrombosis (7). A normal dose (500/kg) of rHuEpo causes a considerable rise in the reticulocyte count within a few days which can be elevated for a week (2). The response to the doses eventually elevates the Hb and Hct which can stay elevated for weeks (1,2). After a treatment of high dose rHuEpo (2000/kg), a lower than normal baseline reticulocyte value can sometimes be the response (2,3). Over time it has been discovered that the administration of a much lower dose of rHuEpo over a longer period of time masks the reticulocyte response. The use of the lower dose reduced the peaks in the reticulocyte count yet still allowed for an eventual increase in the Hb and/or Hct (2).

Anti-doping agencies have developed a number of approaches to the detection of exogenous EPO. Urine sampling for EPO testing of athletes was introduced in 2000 after a method was developed in France (2,7). Differentiating endogenous EPO from rHuEpo administered as a performance enhancing drug has proven difficult and there have been lingering doubts about the ability of this method to differentiate the two types of EPO. Another approach to EPO detection examines the Hb and Hct values of athletes (2). Those with a Hb >170g/L and/or a Hct of >50.5/L are today considered to be involved in EPO doping (10). The OFF-score system was developed to assist in the process of monitoring rHuEpo usage in athletes. A score greater than the ON-model threshold coupled with a concomitant elevated reticulocyte percentage value, suggests current rHuEpo usage. A score that is higher than the OFF-model threshold with a depressed reticulocyte value also is suggestive of recent rHuEpo doping (2,10,11). The initially proposed OFF-model was based on the RetHt value that can only be obtained from the Bayer haematology analysers (2). The “OFF-score” system or “stimulation index” was then reviewed to include the Hb value and the reticulocyte count allowing for universal application of the model by various haematology analysers. The OFF-score is calculated by taking the Hb (g/L) value minus x60 the √ retic % (2,3,11). Scores of over 133 are considered to be evident of doping with the normal range value between 85-95 (2,3). In these studies the reference analyser used for the reticulocyte counts was the ADVIA 120 (2,3). The use of different models of haematology analysers continues to cause interpretation difficulties for using the reticulocyte count as an indication of exogenous rHuEpo usage (2,3).

The OFF-score continues to be used as a screening test for recent use of rHuEpo in athletes. Individual responses to rHuEpo can be quite variable, which may help to explain an occasional disagreement between findings from the OFF-score and other supplementary laboratory results such as urine rHuEpo (2,8). Several studies suggest the detection of gene markers related to erythropoiesis in an athlete's blood might provide more reliable bases for future anti-doping testing (2,8). More recently, an abnormal blood profile score based on the statistical analysis of biomarkers that are indirectly related...
to erythropoiesis has been proposed to detect blood manipulations of both rHuEpo and transfusion in athletes (2).

Conclusions
The reticulocyte count has traditionally been used in medicine as a marker of bone marrow erythropoiesis. In the last decade the reticulocyte count has been applied for use in the detection of illegal blood doping practice among the world’s athletes. By limiting possible pre-analytical and analytical variables, the use of the reticulocyte count in anti-doping testing has become more useful. The comparability of the reticulocyte count derived from differing analysers and applied to sports testing remains in some doubt because of the lack of a standardised reticulocyte calibrator. It has been suggested that this problem could be countered with the application of analyser-specific bias for the reticulocyte calculation offering a more standardised result. Both haemolysis and hypoxia that form with athlete training induces erythropoiesis and improved physical performance at sea level. Illegal blood transfusions and the use of rHuEpo are traceable among athletes and the application of the OFF-score system based on the reticulocyte count is an important tool used by anti-doping agencies in the investigation of performance enhancing drug use. In the field of sports medicine, further research into more accurate methods of identifying athletes undergoing manipulation of the PB red cell mass to improve performance is required.

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