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SEED Haematology

The fight against doping and the role of reticulocyte associated parameters

Athletic performance

Physical fitness and athletic performance, especially in endurance sports, are based on appropriate muscle management. Muscle cells need oxygen for their functionality and a lack in oxygen supply can limit their performance. Consequently, training is not only aimed at learning a clean technique with little energy loss and achieving a general increase in muscle mass, but also at providing the muscles with an optimal supply of oxygen.

There are several ways to achieve this target:

- Cardiovascular training to increase the maximum oxygen uptake by affecting for example the cardiac output which helps to deliver more blood to the exercising muscles.
- Supplying the body with sufficient iron supporting the optimal erythropoiesis.
- Altitude training above 1800 m maximises the number of red blood cells (RBC), in order to supply enough oxygen to the muscle cells.

Impact of altitude training on the body

At high altitude, the air pressure is lower than at sea level. This means that the oxygen is forced into the lungs with less pressure and thus less oxygen is available for loading the RBC. Living and training under these conditions means that the body has less oxygen available. This hypoxia is detected in the kidneys and the body's own erythropoietin (EPO) is released accordingly, in order to accelerate erythropoiesis and thus increase the number of RBC and the haemoglobin content [1]. In this way, the lack of oxygen supply is compensated over time by an increased transport capacity and the muscles are again supplied with the adequate amount of oxygen. Back at a normal elevation, the erythrocytosis results in extra oxygen for the muscles. The muscles perform better which is not only an advantage in a competition but can also boost training performance. After return to a normal altitude the body slowly downregulates the erythropoiesis which can be seen in a decrease of reticulocytes.

Artificial altitude training and blood doping

The overall effect of altitude training is to increase the number of RBC and hence improve oxygen transport capacity, which leads to improved athletic performance. Particularly in the context of competitions, the creation of a training plan before the competition helps to produce this increased performance capacity. Alternatively, a reduction in oxygen supply through an appropriate composition of the breathing air, e.g. during sleep or during training, can lead to similar effects as those produced in real altitude training [2]. This is known as ‘artificial altitude training’.

Another method to increase oxygen transport capacity, which has been used since the 1980s, is known as blood doping [3–6]. On a short-term basis the number of RBC are increased by an autologous blood transfusion (e.g. as a self-donation) [7] or substances whose effects are similar to that of erythropoietin are taken [7–10]. The latter form of doping is also described as 'EPO doping' or 'doping with EPO-like substances'.

EPO doping describes the abuse of a substance that is either identical, **r**ecombinant to the body's own **h**uman **e**rythro**po**ietin (rhEPO), or is equivalent to it in its effect. By taking the substance, the athlete expects positive effects that are comparable to or exceed those of altitude training [5]. Erythropoiesis is stimulated through the intake of EPO-like substances, which results in the availability of additional reticulocytes and consequently more RBC [11].

History of doping with EPO-like substances

Recombinant human EPO became available in Europe in the 1980s, with its usage in sports being prohibited in early 1990. But to enforce this ban, it had to be proven that EPO-like substances had been abused. Direct detection methods were not fully established, especially in the 1990s [12]. Even today, the detection of EPO in urine is still relatively time-consuming and costly [9, 13] for laboratories, with the performance-enhancing effect still being present after the substance has been excreted. Therefore, an indirect detection method for doping with EPO-like substances was developed. This method has examined certain blood parameters and their changes over time (short and long term) since the early 2000s. This procedure was significantly improved by the founding of the World Anti-Doping Agency (WADA) and its establishment of the Athletic Biological Passport (ABP) in 2009 (see Fig. 1) [14].

World Anti-Doping Agency (WADA)

In the summer of 1998, there were numerous doping cases in cycling. The International Olympic Committee took the initiative and promoted the formation of an independent authority in the fight against doping. In 1999, the WADA was established and funded in equal parts by the sporting movement and governments around the world. The most significant achievement is the development and monitoring of the World Anti-Doping Code. This is a document that unifies and standardises all anti-doping efforts in all sports and all countries. To ensure effective compliance with the World Anti-Doping Code, WADA developed the Athletic Biological Passport in 2009.

Further information on WADA, its founding history and its current tasks can be found on the official website.

Detection of blood doping

Since the beginning of blood doping, finding the corresponding proof has been a challenge, as recombinant and endogenous EPO display only minimal differences [12–13, 15]. In addition, there are substances that indirectly enhance the endogenous EPO level or effect, e.g. hypoxia-inducible factor (HIF) stabilisers lead to increased transcription of natural EPO [16]. HIF stabilisers are prohibited, as is the use of rhEPO, but they can only be detected with great effort via liquid chromatography-mass spectrometry [10]. Although direct detection of EPO-like substances is possible nowadays in urine through costly and time-consuming Western blot methods [13], the effect – i.e. the increased number of blood cells – might still be present even if the substance has already been excreted or degraded. Nevertheless, athletes are repeatedly convicted of blood doping. How is this possible?

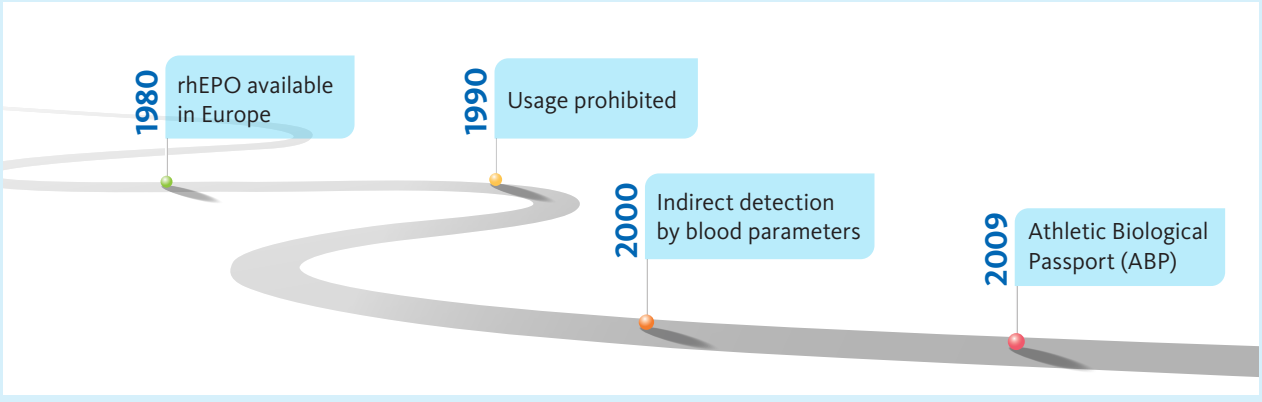

Fig. 1 *History of doping with EPO-like substances*

During the formation of RBC in the bone marrow, nucleated red blood cells develop into reticulocytes which enter the peripheral blood stream.

By degrading the endoplasmic reticulum and its corresponding RNA, the reticulocytes develop into mature RBC within four days. They remain in the bone marrow for three days and within one additional day they mature in the peripheral blood stream [17] to become mature RBC. Through the misuse of EPO-like substances, more reticulocytes are formed and released into the blood. The proportion of these cells increases in relation to the total number of RBC. This increase is exactly what can be measured with a commercially available automated haematology analyser.

The parameter RET% indicates the proportion of reticulocytes to the RBC. RET# indicates the absolute reticulocyte count. In addition to conventional reticulocyte measurement, the fluorescence flow cytometry method allows the classification of reticulocytes into three maturation stages. These maturation stages are defined by the RNA content of the reticulocyte, measured on the analyser as fluorescence intensity. Therefore, additional parameters such as the immature reticulocyte fraction (IRF) can be provided (see Fig. 2) [17].

An unusually high increase in RET% can provide indirect evidence of the use of prohibited substances with EPO-like effects [18].

Any erythropoiesis-stimulating agent triggers a typical pattern of doping including stimulation of reticulocytes. It is followed by increased haemoglobin concentrations and suppressed RET% in response to the artificially elevated haemoglobin levels [19].

Table 1 *Presentation of submitted values per measurement for the ABP haematology module*

Blood variable		Unit
Haemoglobin	HGB	g/dL
Haematocrit	HCT	%
Immature Reticulocyte Fraction	IRF	%
Mean Corpuscular Haemoglobin	MCH	pg
Mean Corpuscular Haemoglobin Concentration	MCHC	g/dL
Mean Corpuscular Volume	MCV	fL
OFF-Score	–	–
Platelets	PLT	10^3/µL
Red Blood Cell Distribution Width	RDW-SD	fL
Red Blood Cells	RBC	10^6/µL
Reticulocytes – in absolute number	RET	10^6/µL
Reticulocyte Percentage	RET%	%
White Blood Cells	WBC	10^3/µL

The monitoring of these parameter changes is supported by the ABP (see info box). National Anti-Doping Organisations carry out a dedicated testing plan to monitor 12 blood parameters per athlete under their local jurisdiction [14, 18]. At regular intervals, the athletes must undergo blood sampling, with the measured values being logged in the ABP (see Table 1). This routine procedure results in individualised threshold values e.g. for the number of reticulocytes in the blood. Simultaneously, additional data are collected and observed over long periods of time and correlated with any changes that may occur in their blood parameters. It is recorded, for example, if high altitude training was taking place or if the athletes were in competition [20].

The ABP can also detect blood withdrawal for a later transfusion, typified by decreased haemoglobin and stimulated RET% and IRF, an approach that proved fruitful in uncovering organised blood transfusion schemes [19].

To allow a definitive conclusion on doping, it must be ensured that the measurement results of the blood parameters are independent of the respective laboratory and its personnel, transport conditions conditions such as time and temperature. It also implies a high degree of accuracy of the haematology analyser.

Athletic Biological Passport (ABP)

The ABP constantly monitors certain biological parameters of athletes. At certain intervals or on certain occasions (e.g. during a competition phase), samples from the athletes are examined in accredited laboratories and recorded in the ABP. In this way, the effect of any abuse of EPO-like substances can be proven over time.

It is up to the respective local anti-doping organisations to integrate the ABP into their individual programmes. WADA has played a leading role in the development of the ABP. The first version in December 2009 already included and used haematologic parameters to establish an athlete-specific profile and thereby detect blood doping. Today, the ABP contains 12 haematologic parameters and a calculated OFF-score. The ABP operating guidelines also specify what needs to be considered when taking samples, as well as how the transport and the analysis itself are to be executed [21].

After the analysis, the values of the blood parameters are collected centrally and processed for evaluation. An Anti-Doping Administration & Management System (ADAMS) is used for this purpose. Its handling is described in detail on the WADA website.

Stability of the reticulocyte parameters

To ensure fair competitions for all athletes, not only the sporting conditions must be comparable, but also the anti-doping controls and the respective measuring conditions in the laboratory. Parameters must be independent of external circumstances. This means on the one hand that even if a sample is measured in different laboratories, at different times of day, at different room temperatures or on different devices in the same analyser series, the measurement result must deliver almost the same, accurate value. On the other hand, the parameters obtained in the laboratory must remain stable even if external influences such as transport times or storage differ.

Many efforts are made by WADA to achieve harmonisation between laboratories such as providing accreditation according to WADA's conditions. WADA developed the world anti-doping code which is a document that harmonises anti-doping policies, rules and regulations within sporting organisations and among public authorities around the world, including testing procedures from sampling, transport and analyses in the laboratory [22]. In addition, studies are carried out to ensure that a possible general difference in measurement is corrected in the ABP, e.g. when changing analyser series [18, 23].

The biological stability has been proven for reticulocyte parameters (RET#, RET%) measured on the XE-Series and the XT-Series in many publications [24–26] and has also been scientifically verified for the XN-Series [25].

added value RET

RET channel measurement technology

In addition to the absolute reticulocyte count (RET#), the use of the fluorescence flow cytometry method in the RET channel provides information on reticulocyte maturation.

The lysis reagent initially perforates the cell membranes while leaving the cells largely native. In a second step, the fluorescence marker labels the intracellular nucleic acids, whereby the intensity of the resulting fluorescence signal is directly proportional to the nucleic acid content. Since the RNA content decreases during the maturation process of the reticulocytes, three parameters can be determined that reflect these maturation stages. Reticulocytes emit a higher fluorescence signal than mature red blood cells, which no longer contain RNA, with a considerably lower fluorescence signal than white blood cells.

According to their fluorescence intensity, reticulocytes are fractionated into three categories, representing different stages of maturity: LFR (low fluorescence ratio), MFR (medium fluorescence ratio), HFR (high fluorescence ratio). The IRF (immature reticulocyte fraction) reflects the proportion of immature reticulocytes and is calculated from the sum of MFR plus HFR.

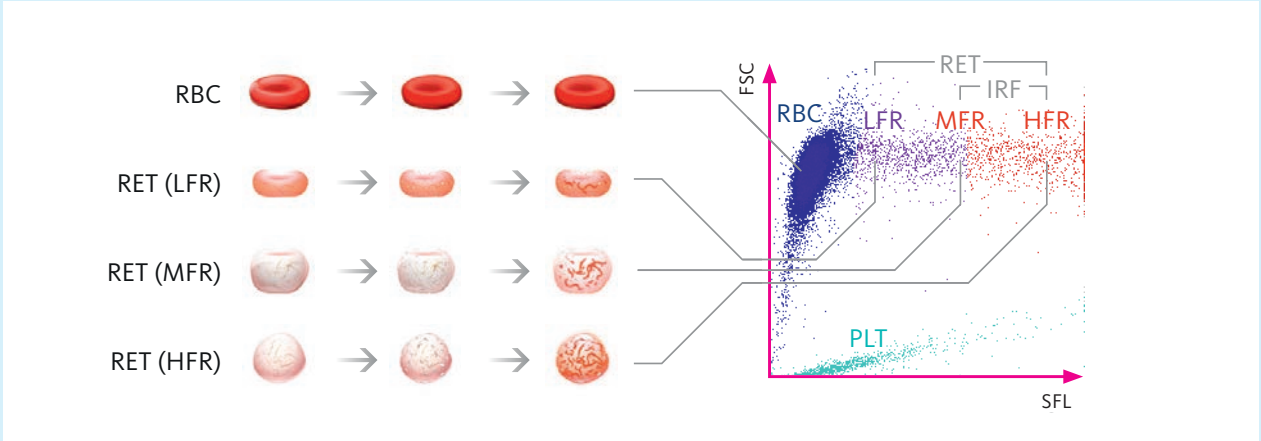

Fig. 2 *RET channel scattergram with maturation stages of reticulocytes, LFR, MFR and HFR*

Conclusion

The fight against doping will always be a race between new substances and the corresponding detection methods. With the help of the reticulocyte, IRF and haemoglobin parameters, however, the status of the erythropoiesis is determined independently of substances. Short-term and long-term monitoring of these parameters provides a scientifically based method of detecting blood doping in this context.

Typical patterns of doping include an increase in RET% triggered by the use of erythropoiesis-stimulating agents, followed by increased HGB concentrations and decreased RET% in response to the artificially elevated HGB levels. The ABP can also detect blood withdrawal for a later transfusion, typified by decreased HGB and increased RET% and IRF.

To ensure a fair doping test, samples used in the ABP can only be analysed in one of the more than 30 WADA-accredited laboratories around the world, all of which use the same analyser technology. The choice of analyser was based largely on the fact that a reduction in analytical variance has a positive impact on the sensitivity of the ABP, where Sysmex analysers demonstrated the lowest analytical variance for RET% [19].

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