The biochemistry of drugs and doping methods used to enhance aerobic sport performance

Chris E. Cooper

Department of Biological Sciences, University of Essex, Wivenhoe Park, Colchester CO4 3SQ, U.K.

Abstract

Optimum performance in aerobic sports performance requires an efficient delivery to, and consumption of, oxygen by the exercising muscle. It is probable that maximal oxygen uptake in the athlete is multifactorial, being shared between cardiac output, blood oxygen content, muscle blood flow, oxygen diffusion from the blood to the cell and mitochondrial content. Of these, raising the blood oxygen content by raising the haematocrit is the simplest acute method to increase oxygen delivery and improve sport performance. Legal means of raising haematocrit include altitude training and hypoxic tents. Illegal means include blood doping and the administration of EPO (erythropoietin). The ability to make EPO by genetic means has resulted in an increase in its availability and use, although it is probable that recent testing methods may have had some impact. Less widely used illegal methods include the use of artificial blood oxygen carriers (the so-called ‘blood substitutes’). In principle these molecules could enhance aerobic sports performance; however, they would be readily detectable in urine and blood tests. An alternative to increasing the blood oxygen content is to increase the amount of oxygen that haemoglobin can deliver. It is possible to do this

1To whom correspondence should be addressed (email ccooper@essex.ac.uk).
by using compounds that right-shift the haemoglobin dissociation curve (e.g. RSR13). There is a compromise between improving oxygen delivery at the muscle and losing oxygen uptake at the lung and it is unclear whether these reagents would enhance the performance of elite athletes. However, given the proven success of blood doping and EPO, attempts to manipulate these pathways are likely to lead to an ongoing battle between the athlete and the drug testers.

**Introduction**

From their earliest lectures, every biochemist learns that oxidative phosphorylation is more ‘efficient’ than mere glycolysis. Increasing your oxygen consumption is good for you, as the popularity of aerobics attests. Yet what to you and I is a desire to increase our oxygen consumption to lose a few pounds is to the elite athlete a career-threatening (if caught) matter of life and death. This chapter will look at the lengths elite athletes will go to increase their aerobic metabolism, with the ultimate aim of improving sports performance. In this chapter I will focus primarily on illegal methods of enhancing blood oxygen delivery at the molecular level i.e. how can increases in oxygen delivery to a cell be enhanced from the vasculature? Thus I will not focus on the hard work and training required to increase cardiac output, muscle blood flow and mitochondrial content; after all this is a chapter about illegally cutting corners! This will also rule out discussion of the more speculative techniques to increase these pathways illegally e.g. targeting VEGF (vascular endothelial growth factor) to improve overall endurance capacity.

I will take an unashamedly biochemical perspective, focusing on mechanism of action and methods of drug detection at the molecular level whenever possible. However, before reaching this point it is clear that we need to at least pay homage to the physiologists; after all the end result of the biochemistry must be to improve the body function so that a person can run faster or for longer.

**Is oxygen limiting for aerobic exercise?**

This question might appear a bit of a tautology, but in fact leads us into a number of controversial areas of current physiology. First a definition: I define aerobic exercise as exercise that is generally performed at levels when blood lactate levels are constant; indeed the ‘lactate threshold’, the amount of oxygen consumed at the point where blood lactate starts to rise, is used as a marker of aerobic fitness. Crudely an increase in lactate arises out of the increase in glycolytic flux due to drops in ATP availability, the ‘Pasteur effect’. This is named after the studies by Louis Pasteur in yeast where he showed that the rate of fermentation was inhibited by oxygen. Current views of this mechanism suggest a role for the phosphorylation of the glycolytic regulator PFK2 (6-phosphofructo-2-kinase) by the AMP-activated protein kinase [1].
The understanding of lactate metabolism in muscle has moved on since the days when it was assumed that lactate passively entered the blood supply on its way to gluconeogenesis in the liver; evidence for direct cell-to-cell lactate cycling in muscle is now convincing [2]. However, the idea that a blood lactate increase represents an inability of oxidative phosphorylation to keep up with increased ATP utilization holds true. It is worth noting that when the media talk about ‘anaerobic exercise’ it is of course nothing of the sort. Except in specialized sports where the exercise itself causes an arterial occlusion (e.g. weight lifting), the flow of oxygen continues maximally and oxygen is still extracted by the mitochondria. But under these conditions the mitochondrial oxidative phosphorylation system, being ‘low power/high efficiency’, needs to be aided by the ‘high power/low efficiency’ glycolytic system.

So the aim of the ‘aerobic’ elite athlete is to maximize mitochondrial oxygen consumption. The maximum rate of whole-body oxygen consumption obtainable during an incremental exercise test, termed $\dot{V}O_{2\text{max}}$, is a marker of aerobic fitness. Take two groups of people, one fit and one unfit, and the latter will have a lower $\dot{V}O_{2\text{max}}$, though interestingly among the elite athletes the ‘lactate threshold’ is generally a better discriminator of actual sports performance [3].

So what limits $\dot{V}O_{2\text{max}}$ and lactate threshold? This is a surprisingly controversial question. One group claim that the maximal theoretical rates of oxygen delivery and metabolism are not directly limiting factors; instead the brain acts as a ‘central governor’ to turn off exercise to prevent ischaemia [4]. This argument has been deconstructed in detail [5], but the controversy never quite goes away. One problem is that the mechanism of the governor (physiological, let alone biochemical) is unclear. Clearly, however, even if the governor was only a minor component, overcoming it would be a dream for every athlete; and we should note that to deny any central component to elite aerobic sports performance is to deny any psychological effects on performance, a clear non-sequitur. Indeed the ‘central governor’ hypothesis can in some senses be thought of as a physio-psychological phenomenon. For example, one biochemical mechanism that has been suggested is that the neurotransmitter 5-hydroxytryptamine, produced during exercise, acts to cause central fatigue in the brain. Some evidence for this comes from the fact that orally administered branched-chain amino acids appear to improve some parameters of endurance performance [6]. The mechanism proposed is that these (entirely legal) dietary supplements compete for tryptophan transport across the blood–brain barrier. As tryptophan is the precursor for 5-hydroxytryptamine synthesis in the brain, this has the potential to lower 5-hydroxytryptamine levels and thus decrease central fatigue.

There is clearly a rich field of neuropsychology and neurochemistry of exercise-induced fatigue waiting to be explored. However, for the rest of the chapter, we will focus on the biochemistry of oxygen delivery and metabolism. Getting oxygen to where it is needed, mitochondrial cytochrome oxidase in muscle cells, can be broken down into a large number of stages, a simplified illustration of which is given in Figure 1.
Increasing $FiO_2$ (the inspired fraction of oxygen) by breathing 100% oxygen can in principle increase $\dot{V}O_2_{\max}$ by raising haemoglobin oxygen saturation. In general during exercise at normal altitude, arterial haemoglobin saturation does not decrease to levels where haemoglobin desaturates, making increases in $FiO_2$ largely redundant. However, in elite athletes the transit time in the lungs is so short that exercise-induced arterial desaturations are possible in some individuals; in these cases exercise performance can be enhanced by raising the $FiO_2$ [7].

Increasing cardiac output and muscle blood flow has the ability to increase the total amount of oxygenated haemoglobin in the capillaries. From there the oxygen transports via diffusion to the cytochrome oxidase. There is an ongoing and lively debate about whether the control of $\dot{V}O_2_{\max}$ is entirely in cardiac output and locomotor muscle blood flow, or includes components of muscle oxygen conductance and mitochondrial oxygen consumption [8,9]. In many ways this argument mirrors the discussion about rate limitation in biochemical pathways that led to the development of metabolic control theory [10]. Here a step (enzyme) could only be 100% rate-limiting (‘bottleneck’) if
a fractional increase in the enzyme concentration caused the same fractional increase in pathway flux. In nearly all metabolic pathways, control was found to be distributed between a number of enzymes i.e. increasing the concentration of a number of different enzymes could increase pathway flux, though in none of the cases was the increase in flux identical with the increase in enzyme concentration. Although this theory has been expanded up the biological hierarchy to whole organs [11] and down to rate constants in single enzymes [12], it is yet to make a significant impact on physiology in general. So, for example, quotations such as those below from a paper reviewing the control of \( \dot{V}_{O_2} \max \) are not atypical [3].

“Their overall conclusion is that \( \dot{V}_{O_2} \max \) is a distributed property, dependent on the interaction of oxygen transport and mitochondrial oxygen uptake. We agree with this conclusion. However, this model cannot determine which of these two factors limits \( \dot{V}_{O_2} \max \) in the intact human performing maximal exertion.” and “However, human studies show that there is only a modest increase in \( \dot{V}_{O_2} \max \) (20–40%) despite a 2.2-fold increase in mitochondrial enzymes. This is consistent with the view that \( \dot{V}_{O_2} \max \) measured during whole-body dynamic exercise, is limited by oxygen delivery (not muscle mitochondria)”. Asking which one factor limits \( \dot{V}_{O_2} \max \) is perhaps the wrong question. Instead a better question would be to ask what is the ‘control coefficient’ of each individual process ranging from 0 (no control) to 1 (complete control). In the latter case the quantitative response would be to say that this establishes that the control strength of the mitochondrial enzymes over \( \dot{V}_{O_2} \max \) is 0.14 (i.e. contains some, but by no means all of the control).

The lack of quantification in control terminology in physiology is certainly hampered by the fact that it is not readily possible in whole organisms to make the kind of precise, graded changes in single steps that allow control coefficients to be more readily measured in isolated cells and organelles. However, it is perhaps illuminating that those involved in modelling exercise [8,13] are the most likely to talk about a ‘distribution of control’ along many of the steps illustrated in Figure 1.

**Is blood oxygen content limiting for aerobic exercise?**

The elite athlete is unlikely to be concerned about the nuances of physiological or metabolic control theory. It is clear that for any exercise lasting longer than a minute aerobic energy systems will predominate. Whatever the exact distribution of the factors controlling maximal aerobic exercise, physiologists are in general agreement that increasing the blood oxygen content will enhance aerobic exercise performance.

Clearly, significantly enhancing the \( pO_2 \) gradient between the vasculature and mitochondria operating close to the lactate threshold will, almost by definition, increase oxygen consumption. Though the precise human study is never easy to do, it has been known since the 1970s that decreasing or increasing total haemoglobin content from baseline (via removal or reinfusion of red
blood cells) can have essentially immediate effects on both $\dot{V}O_{2\text{max}}$ and running performance in the laboratory [14,15] and in the field [16].

From the above it is no surprise that the idea of increasing blood oxygen content has been seen as a route to success in aerobic sports. Historically the sporting authorities have allowed any and all methods that affect the intake of gas into the body, but restricted those that directly add liquids. Put crudely, you can live at abnormally low levels of oxygen (either up a mountain or in a ‘hypoxic tent’), but can’t inject any compound that might have the same effect. Physiology is fine, but biochemistry is a no-no.

**Physiological (legal) methods to improve blood oxygen content**

Here the most prevalent method by far is altitude training. Although one shouldn’t underestimate the psychological benefits (at least for U.K. athletes) of taking a break from the usual dull, wet environment to go to an exotic location which you are told will improve your performance, it is clear that living at altitude affects blood oxygen content directly. The mechanism is well understood [17]. The low $pO_2$ is sensed by cellular oxygen sensors, the best characterized being the non-haem iron enzymes from members of the HIF (hypoxia-inducible factor) family. These trigger the production of the peptide hormone EPO (erythropoietin) in the kidney. This is a global controller of erythropoesis. For an athlete who normally lives at low altitude, going to high altitude (>2000 m) increases total haemoglobin content by about 1% per week [18], with maximal effects requiring as much as 80 days (though this can be reduced if the athlete can tolerate going to significantly higher altitudes). The major problem with altitude training is that, by its very nature, it decreases the availability of oxygen to the tissue. Therefore it is harder to train at altitude. The fact that any effects of increased altitude might be countered by a ‘detraining’ effect has resulted in the ‘live-high train-low’ method: athletes live and sleep at altitude (hours per day) but come to lower altitudes just for daily training. This has been shown to improve performance [19] over the alternative ‘live-high train-high’. The improved performance correlates with the change in haematocrit; indeed the total red blood cell volume increase (8.5%) was almost matched by the increase in $\dot{V}O_{2\text{max}}$ (6%). This suggests that the increase in blood oxygen content is key [20] though this is currently still a matter of active debate [21]. An alternative to the ‘live-high train-low’ hypothesis would be to artificially change your inhaled $pO_2$ in your own environment. Portable altitude simulators were used in the 1980s to force athletes to re-breathe their expired air, thus dropping the inspired $pO_2$. However, this use had been pre-dated by the systematic use of large-scale hypobaric chambers by East German, Scandinavian and, latterly, Australian teams. With the advent of small-scale hypoxic tents dropping the price from hundreds of thousands of dollars to under ten thousand dollars, all elite athletes now have the ability to simulate high altitude at will. Although the theoretical ability of these facilities
in raising haematocrit is clear, performance enhancements are limited. It is probable that this is due to the length of time spent at low $pO_2$. At least 12–16 h is suggested to be necessary to have real performance effects via altitude training [20] making overnight use of the tents for sleeping only unlikely to be productive. The trip to the less-boring mountain resort wins physiologically as well as psychologically!

**Biochemical (illegal) methods to improve blood oxygen content**

Although there has been some ethical debate about the use of ‘hypoxic tents’ by anti-drug organizations (and they were recently banned from Olympic villages), the real way to cheat is to add a foreign compound into your body, especially via injection. Without dwelling too much on the ethics, it is the fact that this can instantaneously, and for no additional work, improve sporting performance that places these compounds and techniques high on the WADA (World Anti-Doping Agency) banned list (Table 1). Red blood cell content can most directly be increased by blood doping. This involves the infusion of packed red blood cells; these can come either from a matched donor or the athlete themselves. In the latter case the phlebotomy is performed a long time earlier allowing the athlete to return to normal haematocrit prior to the infusion. The increase in performance is clear and immediate [15]. However, blood requires a matched donor or the acceptance of some training loss while the athlete recovers from the initial phlebotomy. A far easier, though slower acting, alternative arose when EPO became readily available following the change in its production method, from human cadavers to recombinant techniques. Until out-of-competition drug tests became, at least theoretically, available it is likely that recombinant EPO (rEPO) was widely used by athletes, notably by cyclists in the Tour de France where police seized recombinant drugs from team support workers [22]. The performance effects of rEPO are significant and readily out-perform the use of hypoxic tents; as expected they seem to be mediated by the increase in haematocrit and associated blood oxygen content [23,24].

EPO and blood doping work via enhancing the normal mechanisms of oxygen delivery of the body. However, more exotic compounds are on the WADA banned list. These are the so-called ‘blood substitutes’, a class of compounds that have been in continual clinical development for the last 20 years [25]. They are designed to enhance the oxygen-carrying capacity of blood only; therefore ‘oxygen therapeutics’ is the more correct general term, rather than blood substitute. Figure 2 illustrates the problem. Oxygen, being a neutral hydrophobic molecule, is only poorly soluble in an aqueous phase. Therefore a solution of water in equilibrium with air will contain only approx. 0.2 mM oxygen. The presence of the 10 mM haem in erythrocytic haemoglobin will therefore dramatically enhance the total blood oxygen content by dissolving 50 times more oxygen. Blood substitutes are designed to enhance this rather
Table 1 WADA list of banned doping classes and methods highlighting those that affect blood oxygen content

<table>
<thead>
<tr>
<th>Substance</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1 Anabolic agents</td>
<td>Anabolic androgenic steroids (AAS) Other anabolic agents</td>
</tr>
</tbody>
</table>
| S2 Hormones and related substances | EPO [including other substances with a similar chemical structure or biological effect(s) and its releasing factors]
  | hGH (human growth hormone); IGF-1 (insulin-like growth factor-1); MGF (mechano-growth factor) |
  | Gonadotrophins [LH (luteinizing hormone), hCG (human chorionic gonadotrophin)] |
  | Insulin |
  | Corticotrophins |
| S3 β2 Agonists | Aromatase inhibitors |
| S4 Agents with anti-oestrogenic activity | Selective oestrogen receptor modulators (SERM) Other anti-oestrogenic substances |
| S5 Diuretics and other masking agents | |

Prohibited methods at all times

<table>
<thead>
<tr>
<th>Method</th>
<th>Examples</th>
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<tbody>
<tr>
<td>M1 Enhancement of oxygen transfer</td>
<td>Blood doping (including the use of autologous, homologous or heterologous blood or red blood cell products of any origin) Artificially enhancing the uptake, transport or delivery of oxygen, including, but not limited to, PFCs, RSR13 and modified haemoglobin products (e.g. haemoglobin-based blood substitutes, microencapsulated haemoglobin products)</td>
</tr>
<tr>
<td>M2 Chemical and physical manipulation</td>
<td></td>
</tr>
<tr>
<td>M3 Gene doping</td>
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pathetic oxygen-carrying capacity of plasma. Two main varieties of molecules have been tested, HBOCs (haemoglobin-based oxygen carriers) and PFCs (perfluorocarbons).

HBOCs are, not surprisingly, based on haemoglobin itself [26]. However, putting pure haemoglobin in plasma is not useful as the tetramers will quickly dissolve into dimers/monomers and be cleared via the kidneys. Also, in the absence of the bisphosphoglycerate present in the red blood cell, the
cell-free haemoglobin oxygen affinity ($p_{50}$) is so low that it would never be able to release oxygen. Therefore the biotechnological challenge is to modify haemoglobin such that it remains in the vasculature; this is usually achieved via a combination of chemical cross-linking and conjugation with PEG [poly(ethylene glycol)] derivatives (PEGylation). Fortuitously these changes also modify the $p_{50}$ to close to the physiological range, a generally desirable side-effect, though see Winslow [27] for an alternative view.

A completely different approach is taken by PFCs. These are unreactive hydrophobic molecules that replace the water in plasma. Oxygen solubility
is far higher in PFCs than in plasma; therefore the blood oxygen content is increased by purely physical means. For this reason there is no saturation effect and the oxygen content increases with increases in inhaled oxygen fraction. Therefore clinically PFCs are always used with patients breathing 100% oxygen. This is not necessary for maximum oxygen delivery from HBOCs; provided the oxygen affinity is not too low all the modified haemoglobins will be saturated in the lungs. The ‘money shot’ for PFC efficacy was the demonstration by Clark et al. [28] that there was enough dissolved oxygen in a solution of PFCs for a mouse to be able to swim quite happily completely submerged under water. Hollywood came calling in 1989 when the same demonstration (this time with a rat rather than a mouse) was shown in the movie “The Abyss”; interestingly this scene was censored as ‘inhumane’ in the U.K., but freely passed by the censors elsewhere in the world.

It has been clearly demonstrated that injections of both HBOCs [29] and PFCs [30] increase the blood oxygen content and enhance oxygen utilization in animal models and patients where this has been compromised. However, all else being equal, it is possible that increasing the plasma oxygen content could give a small edge in aerobic exercise, even if the athlete is not breathing 100% oxygen. There are some initial indications that this may indeed be the case for HBOCs [31]. However, these results did not include performance tests and there are theoretical reasons why increasing blood oxygen content outside the normal regulatory control of the red blood cell may be deleterious. A low-affinity PFC or HBOC could release its oxygen too early in the arteriolar system, tricking the muscle into thinking oxygen availability was high and triggering a vasoconstrictive reduction in blood flow [27].

Although they are shown to be able to facilitate oxygen delivery, HBOCs have a number of problems with toxicity. These are related to their radical reactivity. Broadly speaking they kill good radicals and make bad radicals. Outside the protective barrier of the red blood cell, haemoglobin scavenges the vasodilator nitric oxide, causing vasoconstriction; similarly in the absence of the antioxidant defences of the red blood cell haemoglobin is able to initiate free-radical-mediated lipid peroxidation via peroxidative reactions of the haem [32]. This is especially a problem in the kidney, where the HBOC is cleared, and renal toxicity is a not uncommon side effect. Although HBOCs have been engineered to avoid the reactivity with nitric oxide [33], the other toxicity problems have proved less tractable. PFC toxicity has a different mechanism, most probably associated with its immunogenicity as macrophages attempt to clear the foreign particles. Therefore despite over 20 years of development and clinical trials HBOCs and PFCs are not currently considered safe enough for FDA (Food and Drug Administration) approval in the U.S.A. A similar view is held in Western Europe although Hemopure, a product based on modified bovine haemoglobin, has been approved for use in South Africa and Perftoran, a PFC, is available clinically in Russia [34].
Making the most of the oxygen we have got. How do we increase the delivery of oxygen from haemoglobin?

Getting oxygen off haemoglobin
Increasing the oxygen content of the blood is not enough to increase the oxygen content of the mitochondria. The oxygen needs to get from source (haemoglobin) to cell to organelle. The first part of this process (offloading oxygen from haemoglobin) has been of interest to athletes recently. The simple binding of a single ligand to a protein occurs via a hyperbolic binding curve. This is the case when myoglobin binds oxygen for example. However, the four oxygen molecules bind to the tetrameric haemoglobin co-operatively, leading to the sigmoidal binding curve beloved of undergraduate biochemistry students (Figure 3A). This shape of the oxygen dissociation curve allows more oxygen to be delivered from lung to cell than an equivalent hyperbolic non-cooperative binding curve (see Figure 3A). It can be calculated (Mike Wilson, personal communication) that the amount of oxygen delivered is maximal when the haemoglobin \( p_{50} \) is at the geometric (not the more common arithmetic) mean of the lung and tissue \( p_{O_2} \). Given that haemoglobin has a \( p_{50} \) of 27 mmHg and alveolar \( p_{O_2} \) is about 100 mmHg, this means that haemoglobin has evolved to deliver oxygen maximally at a capillary \( p_{O_2} \) of 7.5 mmHg (Figure 3B). Under these conditions the haemoglobin would be about 5% saturated with oxygen; consistent with this theory elite athletes can indeed dramatically desaturate their haemoglobin (<10%) when passing through a highly active muscle bed [35].

So, given the above, is there a way to increase the offloading of oxygen from haemoglobin, either to bring ‘normals’ into the elite range or give elite athletes an extra edge? There is some evidence that this may be possible by shifting the haemoglobin dissociation curve. A number of molecules bind to haemoglobin and alter its oxygen affinity (generally by stabilizing the low affinity T state and hence right-shifting the haemoglobin dissociation curve). These include carbon dioxide, protons and bisphosphoglycerate. In the absence of bisphosphoglycerate, for example, haemoglobin would bind oxygen almost as tightly as myoglobin and it would be impossible to offload oxygen to tissue at all. Indeed the first thing that happens when someone goes to altitude is that the bisphosphoglycerate concentration increases in their red blood cells. This increases oxygen offloading in the tissue, at the expense of decreasing the saturation in the lung (clearly a delicate balance, see Figure 3C). All right-shifting reagents have the same problem; any increase in oxygen dissociation in the tissue risks compromising the reloading of oxygen on to deoxyhaemoglobin in the lungs. The one exception is the humble proton. A muscle exercising at its maximal capacity will become acidic. The acidification of haemoglobin will right-shift its \( p_{50} \) resulting in the delivery of more oxygen. However, it will have no effect on oxygen-binding in the lungs as the pH is neutral, yielding the best of both worlds (Figure 3C).
Figure 3. The haemoglobin dissociation curve and oxygen delivery

(A) The extra oxygen that can be delivered to tissue due to the sigmoidal-binding curve resulting from the allosteric nature of oxygen binding. (B) Illustration of the maximum amount of oxygen that can be delivered to tissue [the oxygen p50 being the geometric mean of the alveolar and tissue (microvascular) pO2]. The low tissue pO2 can be achieved in exercising muscle of an elite athlete. (C) The effect of right-shifting the haemoglobin-dissociation curve; less oxygen can be picked up in the lungs, but more oxygen can be delivered to the tissue.

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There is one compound on the WADA banned list that does indeed affect the haemoglobin dissociation curve. RSR13 (right-shifting reagent 13 or faproxiral) was designed to release more oxygen to tumours; the more oxygen that is present the more effective radiation therapy is in creating toxic oxygen radicals. In animal studies it has been shown to increase $\dot{V}O_{2\text{max}}$ across an electrically stimulated canine gastrocnemius muscle [36]. However, in these studies the arterial oxygen saturation was kept artificially high by breathing on 100% oxygen. Unlike the proton, RSR13 will be present in the lung and muscle vasculature. It is unclear in a human athlete at normal $pO_2$, whether the gain at the bottom of the haemoglobin dissociation curve would offset the loss at the top (Figure 3C).

**Getting oxygen from the red blood cell to the endothelium**

Once off the haemoglobin, oxygen still has to get to the muscle mitochondria. Inside the muscle cell it is well-characterized that a major physiological function of myoglobin is to increase oxygen diffusivity; it is faster for oxygen to diffuse via binding and rebinding to myoglobin molecules than to move through an aqueous solution. Could the same thing be possible in plasma? It is clear that oxygen diffusion in plasma is limited by its poor diffusion through water. Putting a rapidly diffusing molecule into this solution that can increase the concentration of oxygen will enhance oxygen transport through the plasma. In this case, like myoglobin, the protein is not acting primarily to increase the blood oxygen content, but instead is increasing the oxygen diffusion through an aqueous phase. Indeed it seems possible that one new haemoglobin-based blood substitute, MP4 [37], may, at least in part, act via such a mechanism. It is unlikely that the PFC-based blood substitutes would have the same effect as they form an emulsion in the plasma which raises a barrier to diffusion. Whether any effect of low dose HBOCs on improving plasma oxygen transport in healthy elite athletes would improve performance depends, of course, on how much this diffusivity limits oxygen delivery under these conditions; the sporting environment is very different to those in a clinical trial of blood substitutes.

**Evidence in the field? What techniques are used to enhance oxygen delivery to tissue?**

Most of the techniques described have indeed been used by athletes in competition. On the legal front, altitude training is a major training component of most elite athletes involved in long-term endurance events. Hypoxic tents for individuals (or more rarely hypoxic rooms for groups) are less common, but still a frequent occurrence. Of the current ‘illegal’ methods, blood doping came to prominence in the 1970s and 1980s with both Finnish and Italian distance runners and the 1984 U.S. Olympic cycling team admitting to its use; blood doping was not formally prohibited at the time, although the revelations were treated with considerable disquiet.
Since then blood doping has apparently taken a back seat to EPO, but more sensitive testing has revealed that it has not gone away. Homologous, rather than autologous, doping seems to be the order of the day, given that there is then no loss in training during the recovery period. There are persistent rumours of ‘ringers’ sent to games merely to provide blood for the elite athletes (even in some cases suggestions that weaker members of teams have been specifically selected as blood donors for the ‘team leader’). Equipment consistent with the use of blood transfusion has been confiscated from the Austrian team at both of the last two winter Olympics (2002 and 2006). Recently Tyler Hamilton, the gold medallist in the 2004 Olympic men’s individual cycling time trial, was found guilty of homologous blood transfer and banned from cycling for 2 years.

Though it is likely to have been taken much earlier, the use of EPO came to prominence in the 1998 Tour de France when samples were found in the car of the Festina team’s soigneur, Willy Voet [22]. The Chinese authorities withdrew a number of their rowers from the Sydney 2000 Olympics after positive EPO tests prior to the games and two track athletes, Zheng Yongji and Li Huiquan, were expelled from the 5th Chinese City Games in 2003. A 3000 m steeplechaser (Brahim Boulami in 2002) and an elite cyclist (Roberto Heras in 2005) have also both fallen foul of the recently designed EPO test [38]. Derivatives of EPO have not escaped the attention of athletes. Darbepoetin is a synthetic glycopeptide that acts via the EPO receptor; two additional N-glycosylation sites with up to 25 sialic acid residues extend its half-life in the serum and increase its potency compared with EPO. Darbepoetin is used to treat anaemia associated with chronic renal insufficiency and chemotherapy [39]. Russian (Olga Danilova and Larissa Lazutina) and Spanish (Johann Muehlegg) cross-country skiers and Russian (Faat Zakirov) and Italian (Roberto Sgambelluri) cyclists all tested positive for darbepoetin in 2002.

There is no evidence that the haemoglobin modifier RSR13 has been used in sport. However, indirect evidence suggests that some athletes may have been attempting to use blood substitutes. Given the toxicity of both fluoro- carbon- and haemoglobin-based blood substitutes, it is perhaps just as well that these incidences seem to have been isolated, if indeed they occurred at all. My favourite story in this regard concerns the cyclist Dario Frigo at the 2001 Giro d’Italia. Two vials of the first-generation blood substitute, Hemassist™, were confiscated in raids by police and he was ejected from the race and sacked by his team. Found guilty on other doping charges Frigo admitted that he had bought the haemoglobin-based blood substitute on the Internet. Although he could have been forgiven for not knowing that this compound is best kept in a frozen state until immediately prior to use, the fact that it was colourless might have been a give away! The solution, which Frigo never took, was later shown to be saline.
Is oxygen doping risk-free? Catching the crooks

Given the list of doping agents and techniques WADA list in this area it is clearly necessary to have a wide range of tests. The major physiological effect of blood doping and EPO treatment is an increase in haematocrit. Indeed the simplest test is to test all athletes prior to an event and refuse to allow entry if the haematocrit or haemoglobin content is above a defined safe level e.g. 50% for haematocrit in males and 47% in females. This test is still used in some sports, notably cycling and cross-country skiing. No further fault is attached to this failure, although it can trigger a urine test for EPO. A justification for this can be made on safety grounds, given the concerns about the possible dramatic effects of blood thickening on the circulation. The exact level of this test, high enough to deter cheats but not catch innocent athletes using altitude training, is continually controversial. It is also clear that some athletes have a genetically abnormal haematocrit. Although this is an allowed exception it does require samples from childhood (not held by many people!) or the hope that other family members have the same condition. The oft-quoted example here is Eero Mantyranta, a Finnish cross-country skier who won gold medals in the 1964 Winter Olympics; along with the rest of his family, Mantyranta was shown to have a mutation increasing the activity of his EPO receptor (EPO-R).

One problem with this kind of test is that it is so easy to ‘do-it-yourself’. Clearly the ease of the test is a benefit in that the official testing can be done just before a race. However, it also allows athletes (or their team) to adjust the haematocrit to end up just below the 50% threshold, while still gaining much of the benefits from blood doping or EPO. This test relies on a link between haematocrit and blood oxygen content. In general, increasing the haematocrit will lead to an increased red blood cell volume, increased total haemoglobin concentration and hence an increased number of sites for oxygen binding. This is generally true for chronic changes. However, haematocrit is not always linked with the number of oxygen-binding sites. Acute dehydration can lower blood volume, with a consequent increase in haematocrit. However, the total blood haemoglobin content, and hence the number of oxygen-binding sites, remains unchanged. Conversely a 500 ml saline infusion can potentially increase blood volume enough to drop the haematocrit and pass the test. However, there will be minimal loss of the illegal enhanced ability to carry oxygen.

Some teams go to extremes to pass this test. Six members of the Finnish ski team used the plasma volume expander HES (hydroxyethyl starch) prior to the world Nordic ski championships in 2001. HES is banned; raising plasma volume can potentially be performance enhancing and, as we have seen above, its use can mask a rise in haematocrit caused by illegal methods. Unfortunately for the Finnish team, a test had recently been developed for HES (and not advertised), resulting in the string of positive tests.
Direct tests for homologous blood doping are clearly possible, given that a large amount of a foreign blood has to be injected for it to work. The small amount of DNA in preparations of packed red blood cells means that tests have focused on the cell surface. Fluorescence-activated cell sorting is used to detect antigens different to the athletes’ normal blood (in effect making use of blood group antigens that are not significant enough to prevent a healthy transfusion). If these antigens can be proved to exist, then genetic exceptions to this procedure are very rare. Most are likely to preclude sport (or be well-known) e.g. bone marrow transplants. One possibility that gets the media very excited is that someone who is naturally chimaeric (i.e. who had a ‘vanishing twin’) would test positive. However, this can be readily checked. Tyler Hamilton (see previously) was the first person to fall foul of this test. His appeal was recently rejected by the Court of Arbitration in Sport, although he still protests his innocence.

Tests for autologous blood transfusions are clearly much more difficult. As autologous blood doping uses blood removed many weeks prior to the event, one possibility is to detect for the changes occurring during freezing or extended refrigeration of the red blood cells.

The biggest advance in recent years has been the development of tests for EPO [38]. These consist of a short-term urine test that can only detect recent use (as EPO is only stable for a few days) and a longer-term blood test designed to determine whether excessive red blood cell development has occurred recently. Testing for illegal EPO use is problematic as there is a need to distinguish injected peptide from that produced naturally in the body. The urine test takes advantage of the fact that the human EPO that is readily available for athletes is the recombinant form made in *Escherichia coli*. Therefore the pattern of glycosylation of the EPO isoforms is different from human EPO made in a human cell. Isoelectric and immunoblotting are used to distinguish between endogenous and recombinant human EPO [40]. The EPO test has been criticised as it is fundamentally different from the more standard drug tests such as GC–MS that look to detect a unique foreign molecule. The question revolves not around false negatives, but false positives. Even a 1 in 1000 false positive is likely to affect someone’s career unfairly as the test is widely used. Recently (2005) the Belgian triathlete Rutger Beke was found to naturally excrete proteins following intense exercise that gave a positive EPO test; his conviction was overturned by the Belgian authorities. The culprit seemed to be α1-antichymotrypsin which was able to bind to the EPO antibodies [41]. How common this pattern is remains unclear (and therefore how often it could lead to false positives is unknown). Beke’s exercise-induced hypoproteinuria needed to be coupled to bacterial contamination of the urine sample post-test to deliver a positive EPO result; it is likely that a current properly conducted EPO test would not show this artefact.

The blood test for EPO has a completely different basis. It looks at patterns in a variety of blood parameters to see whether indications of drug abuse
can be observed. Different models are used to detect increasing (ON) and decreasing (OFF) rates of erythropoiesis. The initial test [42] used haematocrit, reticulocyte haematocrit (i.e. number of immature erythrocytes), percentage of macrocytes, serum EPO and soluble transferrin receptor (an indicator of the iron production required for erythropoiesis). Second-generation tests were improved by only including characteristics independent of red blood cell volume, namely haemoglobin concentration, percentage of reticulocytes, serum transferrin receptor and serum EPO concentrations [43]. The blood test is indicative of drug use only and needs to be coupled with a urine test for an athlete to be found guilty. Nevertheless, although appearing on the surface to be less specific than the urine EPO test, these multi-parameter tests have the potential advantage in the long run of being able to detect the abuse of any compound that increases red blood cell development (even ones not yet known to the authorities).

The abuse of the other compounds mentioned in this chapter is much easier to detect. Darbepoetin does not suffer from similarity to a normal human peptide. Although it is currently detected by the EPO urine test, it would not be impossible to devise a more specific test if this proved necessary. Although there is no current validated test for haemoglobin-based blood substitutes, HBOCs exist outside the red blood cell; therefore the presence of plasma looking like a strawberry milkshake would be a bit of a give-away. Likewise PFCs exist as an emulsion, so in this case the milkshake would be vanilla. These assays could be readily worked up scientifically if required [44,45]. RSR13 is not so obvious to the human eye, but tests have already been developed using GC–MS [46].

The future

What does the future hold for oxygen doping? It is clear that these methods can provide significant performance enhancements. Therefore the history of sport tells us that there will be an ongoing struggle between athletes trying to bend/break the rules and drug testers trying to catch them. The appearance of the designer steroid [THG (tetrahydrogestrinone)] not used in clinical medicine has opened up the whole doping field to new problems. No longer is it necessary to just predict which new pharmaceutical products might be developed by athletes and develop appropriate tests. Compounds only developed for sport are likely to be produced. Apart from the obvious (more EPO derivatives) there is likely to be a whole panopoly of compounds that will affect red blood cell development of which we are as yet unaware. For example, activation of HIF will trigger erythropoiesis with no requirement for external EPO. Even in the case of EPO, the latest recombinant EPOs are being made in human cell lines; the glycosylation patterns are therefore likely to be identical with ‘normal’ EPO, rendering the current EPO urine test obsolete. Similar problems will exist if (once?) gene doping of EPO becomes possible;
although the health effects of a permanently turned on EPO response, and/or possible down-regulation of responses, make the latter more than just a problem in genetics.

In the light of these issues the strategy of the SIAB (Science and Industry Against Blood Doping) research consortium is worth noting. This includes a strong intention to develop new tests of the biological markers for blood doping, especially important given that the success of EPO testing may mean that 'old-fashioned' blood doping may once again become fashionable. Tests could, in the long-term for example, include transcriptomics and proteomics assays, once these become cost-effective for routine use. The use of a 'Haematologic Passport', where the athletes normal blood values (especially haemoglobin concentration and reticulocyte content) would be known in order to compare changes observed during an event, would reduce many of the variables, and complaints, surrounding current and future blood tests.

We have seen that blood oxygen content is only one of many determinants of aerobic sports performance. However, it is probably the one that is easiest to manipulate pharmacologically and physiologically. It seems possible to develop tests to ensure a level playing field at the start of a race where no athlete has an unfair advantage in terms of the amount of oxygen (in the blood) they can deliver to their tissue. Whether it is possible ever to be absolutely sure that all athletes worked as hard to get to this ‘oxygen-rich’ place is a more difficult question to answer.

Summary

- **Sport performance for events lasting longer than 1 min (aerobic) is predominantly powered by energy derived from mitochondrial oxygen consumption.**
- **Mitochondrial oxygen consumption is maximally limited, at least in part, by oxygen delivery to the muscles.**
- **Blood oxygen content increases oxygen delivery and therefore maximal oxygen consumption.**
- **In most, but not all, cases arterial haemoglobin is always fully saturated with oxygen during exercise.**
- **Therefore an easy, and proven, way to increase aerobic sports performance is by increasing the total blood concentration of haemoglobin.**
- **Legal methods to do this are via altitude training or hypoxic tents.**
- **Illegal methods include blood doping or injections of erythropoietin (EPO); good evidence exists that these methods enhance aerobic sports performance.**
- **More experimental methods of enhancing oxygen performance include blood substitutes and reagents to alter the haemoglobin oxygen dissociation curve; there are no human studies showing that these methods enhance aerobic performance.**
• EPO injections and other methods to enhance oxygen transfer are banned by WADA.
• Methods exist to test for the presence of recombinant EPO in urine, but the efficacy of this test is limited by the short half-life of EPO and its long-lasting physiological effect.
• There is an ongoing development of direct and indirect blood tests for EPO and blood doping.

References

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