Review

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Detection methods for autologous blood doping

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The use of blood doping is forbidden by the World Anti-Doping Agency. Several practices, such as blood transfusions are used to increase oxygen delivery to muscles and all of them are highly pursued. In this regard, the development of accurate methodologies for detecting these prohibited practices is one of the current aims of the anti-doping control laboratories. Flow cytometry methods are able to detect allogeneic blood transfusions but there is no official methodology available to detect autologous blood transfusions.

This paper reviews protocols, including the Athlete Biological Passport, that use indirect markers to detect misuse of blood transfusions, especially autologous blood transfusions. The methods of total haemoglobin mass measurements and the detection of metabolites of blood bags plasticizers in urine are reviewed. The latter seems to be an important step forward because it is a fast screening method and it is based on urine, a fluid widely available for doping control. Other innovative approaches to blood transfusion detection are also mentioned. A combination of the reported methodologies and the implementation of the Athlete Biological Passport is becoming a promising approach. Copyright © 2012 John Wiley & Sons, Ltd.

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Introduction

Increasing the oxygen arriving to the exercising muscle is one of the more powerful means of performance improvement, especially in aerobic sports. There are several means of reaching this goal, either by substances that may alter the haemoglobin-oxygen saturation curve (e.g. efaproxiral[1]), or by using new oxygen carriers either based on haemoglobin (HBOCs)[2] or other chemicals (e.g. perfluorocarbons).[3] A steady increase of red blood cells (RBC) count may be also obtained by erythropoietic stimulant agents such as erythropoietin or related drugs.[4–7]

Often, however, athletes search for an acute increase in RBC by means of blood transfusions.[5] Flow cytometry methods for membrane surface double population of antigens may reveal the homologous (or allogeneic) blood transfusion approach. Of special concern is the increasing transfusion of the athlete's own blood or red cell concentrates (autologous) as compared with the more easily detectable homologous blood-doping practices, because transfused RBCs bear the same surface antigens as the other RBCs in the athlete's body, being undetectable by the above-mentioned analytical approach.[8–12] Thus, ingenious approaches for indirect blood markers, including total haemoglobin mass measurements, or to test for the excretion of metabolites of bag plasticizers in urine are new proposals for detecting these prohibited practices. In fact, some police raids have identified networks of medical or paramedical people helping athletes to store and re-infuse their own stored blood.[13]

Blood transfusion gives rise to alterations in erythropoiesis and therefore the possibility exists for the measurement of changes in some indirect markers which can complement direct evidence of these malpractices in sport. The more recent approach of using indirect markers, mainly based on blood parameters, will be reviewed. On the other hand, the highly promising use of urine analysis to detect plasticizers originated in the blood-containing bags and absorbed into the recipient body will be also summarized. The excretion of their metabolites offers new insights into the capability of detection of this prohibited method. Lastly, alternative approaches that may be relevant in the future for autologous blood transfusion detection are also mentioned below.

Blood markers approach

After the pioneering work[14] where physical performance was clearly directly related to Haemoglobin concentration (Hb) in subjects submitted to blood loss and re-infusion, the practical benefit of blood transfusion in several sport disciplines was rapidly recognized.[15–19] The revelation by the US Olympic Committee[20] that seven members of the Olympic cycling team had received blood transfusions, prompted critical reaction from the American College of Sports Medicine[21] indicating that the use of blood doping as an ergogenic aid for athletic competition was unethical and unjustifiable. On the eve of the Winter Olympic Games in Calgary in 1988, the International Olympic Committee (IOC) Medical Commission issued a statement on the banning of such practices.[22] It recognized that the simple determination of Hb in blood would not fully discriminate innocent from guilty athletes. They emphasized the interest of another pilot research carried out based on the analysis of two blood samples, one before and one after competition, but clarified that the validity of the test was still too low for application at that time. Such work[23] was mainly carried out studying six elite cross-country skiers, who were phlebotomized and re-transfused with three units of own blood four weeks later. If the two-sample approach was used, an increase in Hb of more than 5% and a decrease in serum erythropoietin

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(EPO) by more than 50% appeared to discriminate more than 50% of blood-doped athletes.

Several other possibilities were later proposed in the literature concerning the detection of autologous blood transfusion, most of them based also on measurement of blood parameters.

It is known that the phlebotomy process induces changes in markers of erythropoiesis as a result of the temporary situation of anaemia. Some of the blood parameters, such as those previously mentioned – Hb, serum erythorpoietin (sEPO) – react very quickly. Others – percentage of reticulocytes (retic%), serum transferrin (sTfR) – react slowly. For Hb in particular, a difference of around 15% could be expected between the anaemic phase and the recovered period several weeks later, if three units of blood are being re-infused.[23,24] It is difficult to find an alternative explanation to the donation of blood to give rise to this big a difference; therefore it is realistic to suspect that someone is trying to continue his/her own blood re-infusion. However, the sensitivity of the approach would be decreased if the amount of blood re-infused was lower (one or two units).

When blood already donated is re-infused in autologous blood transfusion, major changes are evident for several blood parameters. As already mentioned, if two blood samples before and after the blood transfusion process separated by more than 15 days are obtained from the same subject, changes in haematocrit (Hct), Hb and OFFhr (Hb-60(%retic)$^{1/2}$) are observed, which were again proposed as the basis for this type of doping detection.[25] However, as some of these parameters are easily affected by dehydration or altitude training, the use of this protocol seems limited.[26] New parameters, such as the relationship of total RBC haemoglobin (RBCHb)/total reticulocytes haemoglobin (reticHb), have been proposed for greater sensitivity.[27] This relationship has the advantage that variations in plasma volume affect the numerator and denominator similarly and should cancel the effect on the scoring of the test. The re-infusion of blood originated supra-physiological concentrations of RBCHb and reduced RetHb, resulting in an increase in the proportion RBCHb/reticHb. By extending the study period to several weeks after the autologous blood transfusion,[28] then the score OFFhr appears as an alternative useful potential indicator, but without sensitivity in acute phases after the re-infusion of blood. Given that the ergogenic benefit for athletes who receive autologous blood transfusion appears shortly after re-infusion, acute insensitivity is a serious drawback.

From studies of the different blood parameters as an acute indicator, so far the total mass of hemoglobin (tHB) seems the most sensitive.[26,28–31] Initial studies done with only two days between blood donations and blood re-infusion[30] showed the suitability of the approach. However, when the method was extended to a more normal practice (longer period between the two procedures), the method presented sensitivity yet, but showed the limitation of the need for prior knowledge of the stable baseline for each athlete, which is quite difficult. As such, it seems more as an additional tool than as an anti-doping method by itself. The fact that the test requires re-breathing carbon monoxide (CO)[31] in an experimental setting poses additional constraints for routine application for two reasons. First, CO is considered toxic and transiently reduces exercise capacity. Second, the full cooperation of the athletes with the testing officer is needed, which is improbable in cases of subjects who cheat.

Some of the drawbacks mentioned above can be counteracted by the application of longitudinal tracking of markers under the scheme of an Athlete Biological Passport (ABP).[32] Therefore, in order to minimize sources of variation, an intra-individual longitudinal evaluation of haematological parameters was proposed. A reference range specific for each subject should be investigated. Preliminary results of Hb, retic%, and OFFhr when subjected to a model based on Bayesian statistics showed a significant sensitivity (82% by combination of different parameters).[33] The haematological parameters selected for the ABP by the World Anti-doping Agency (WADA)[32] are: RBC, mean cell volume (MCV), haematocrit (HCT), Hb, mean cell haemoglobin concentration (MCHC), retic%, reticulocytes count (ret#), mean cell haemoglobin (MCH), and OFFhr. Interestingly, the stability of reticulocytes and haemoglobin in male triathletes' profiles for several consecutive years has been reported recently.[34] In the same study, apparent differences were observed between genders for some parameters and greater variability in female athletes. Strict protocols were studied for the collection, transportation, and analysis of the samples in an attempt to optimize the robustness, and the WADA made those regulations mandatory.[32,35]

Plasticizers in urine

A promising method was developed to detect blood transfusion misuse, based on the measurement of the metabolites of the plasticizer di-ethylhexylphthalate (DEHP) in urine.[36–38] The use of DEHP has been extended in medical devices, especially in the bags used to store blood products, which have been authorized for the last three decades. The good preservation conditions of blood and its components are the main benefits of using DEHP in the bags,[39–42] although there is a high exposition to this chemical during the transfusion process.

DEHP has endocrine-disrupting properties and it can specially affect the male reproductive system. The organs highly affected in the animals studied were liver, thyroid gland, kidneys, and testis.[43,44] Regarding mutagenicity and carcinogenicity studies, DEHP was classified as a non-carcinogenic substance for humans.[45] The presence of DEHP in a large amount of products[46,47] implies continuous exposure of the population.[48–51] Some populations are more exposed than others; for example, patients receiving blood transfusions, dialysis, extracorporeal membrane oxygenation and neonates receiving different kinds of treatments whose levels may be five-fold higher than the allowed daily tolerable intake.[52]

Because of epidemiological and toxicological purposes, the metabolism of DEHP in humans has been evaluated in different studies after oral doses of tetra-deuterated labelled DEHP.[53] DEHP is metabolized to different metabolites. In the first step, DEHP quickly becomes the monoester mono-(2-ethylhexyl) phthalate (MEHP) and 2-ethylhexanol by the action of the lipase enzymes in the gastrointestinal tract. Then, MEHP is metabolized to different oxidized compounds in the liver and they are excreted in the urine because they do not accumulate in the body due to their chemical properties. The main metabolites in the first 24 h after exposure are the oxidized metabolites mono-(2-ethyl-5-hydroxyhexyl)phthalate (MEHHP), mono-(2-ethyl-5-oxohexyl)phthalate (MEOHP), mono-(2-ethyl-5-carboxypentyl) phthalate (5cx-MEPP) and mono-(2-carboxymethylhexyl)phthalate (2cx-MMHP).[54] The metabolites with the longest half-life are 5cx-MEPP and 2cx-MMHP, while MEHP has the fastest elimination pattern.[55–57] Due to DEHP contamination, these metabolites are

present in the urine of the general population, but markedly high concentrations of the metabolites are only found in urine of highly exposed people.

The first evidence showing that DEHP metabolites could be used as markers of blood transfusion was described by Monfort *et al*.[36] It was hypothesized that subjects submitted to blood transfusions are exposed to the plasticizer DEHP present in the bags used to store blood. Concentrations of the metabolites MEHP, MEHHP, and MEOHP were measured in four population groups: control group with normal exposure to DEHP, hospitalized patients receiving blood transfusions, non-transfused hospitalized patients receiving other medical care involving plastic materials, and athletes. Low concentrations of DEHP metabolites were obtained in urine samples of the control group (range of mean values for each metabolite at 90th percentile 27–76 ng/ml), and the athletes group (range of mean values for each metabolite at 90th percentile 20–52 ng/ml). Urinary concentrations of all three DEHP metabolites were significantly higher in patients receiving blood transfusions (range of mean values for each metabolite at 90th percentile 620–925 ng/ml, range of maxima 2362–5174 ng/ml). Thus, elevated concentrations of DEHP metabolites in urine indicated an increased exposure to DEHP, such as that occurred in blood transfusions, and it was concluded that these metabolites could be used as markers for suspected blood transfusion misuse.

These results were corroborated in other studies. Solymos *et al*. [37] measured the same DEHP metabolites (MEHP, MEHHP, MEOHP) in a control group, in hospitalized patients receiving blood transfusions and in athletes. The investigation also demonstrated that significantly increased levels of these DEHP metabolites were found in urine samples of transfused patients, strongly indicating blood transfusion.

The confirmation that DEHP metabolites in urine can be used as markers of the misuse of blood transfusion is shown in an experiment of autologous blood transfusion recently published.[38] The study was performed with 25 moderately trained subjects. The protocol consisted of blood collection from all subjects, preparation of RBC concentrates from the blood collected, storage of the RBC concentrates at 4 °C for 14 or 28 days, and re-infusion of the RBC concentrates. The results indicated that the three metabolites monitored had an important increase a few hours after the transfusion and additionally, these high levels were observed even the day after. The practical detection period would be 1–2 days after blood transfusion and an example of the results obtained for one of the metabolites is presented in Figure 1. Moreover, the concentration of the three metabolites

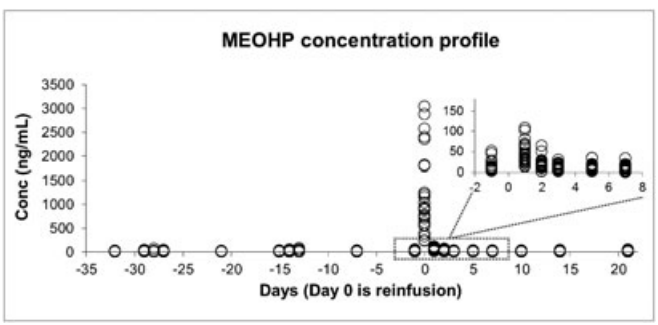

Figure 1. Concentrations in urine (adjusted to a specific-gravity of 1.020) of one of the metabolites of DEHP in 13 subjects receiving one unit of their own blood (day 0) previously donated (day −28) and stored refrigerated for 4 weeks. For details, see Montfort *et al*.[38]

tended to be related to the storage time of the RBC as higher concentrations were detected after longer storage periods.

The measurement of DEHP metabolites allows screening for blood transfusion misuse using urine samples, which are always available in the doping control tests. In addition, it may be useful for both autologous and allogenic transfusion. Compared to other tests that are performed for blood-doping detection (such as flow cytometry, used to detect allogenic transfusion), the method proposed is cheaper, less time-consuming, and easy to perform because it is based on liquid chromatography coupled to tandem mass spectrometry technology, nowadays available in all anti-doping control laboratories. However, more work is needed to evaluate if other DEHP metabolites as 5cx-MEPP and 2cx-MMHP could be also used. Preliminary studies suggest that they are good markers and they could extend the detection window for a few hours because they have the longest half-life of elimination, especially 2cx-MMHP. In addition to the concentration of DEHP metabolites, it was observed[38] that the ratio between some of the metabolites[57] may be also used to increase the detection window.

Nevertheless, the possibility of other sources of high DEHP exposure cannot be completely excluded, as unexpected high concentrations of the metabolites were also found to be as result of occupational or diet occasional exposures.[58,59] In this regard, longitudinal studies are needed to identify the possible cause of some unexpected increases. The basal levels of DEHP metabolites may be incorporated as a part of the individual ABP, to monitor possible changes in the individual urine concentrations which should allow suspecting for the misuse of blood transfusions.

PVC plasticized with DEHP is the most common material used for storage of RBC-containing blood products. Testing for DEHP may speed up the adoption of alternatives (e.g. materials free of DEHP) among doping athletes, as normally occurs for other doping substances and methods. Up to now, there is no available alternative for blood storage which offers the same qualities as DEHP-containing materials. It preserves the whole blood as well as its different components – RBC, white blood cells, plasma, and platelets – in good conditions. Different plasticizers and several polymers have been studied as alternatives to DEHP for blood bags, but shorter storage times were obtained for the RBC[60] which indicates that a replacement is not imminent.

Other promising approaches

It is well known that RBC undergoes significant alterations during storage.[61–63] These alterations include morphological changes, slowed metabolism with a decrease in the concentration of adenosine triphosphate, acidosis with a decrease in the concentration of 2,3-diphosphoglycerate, loss of function of cation pumps, oxidative damage of proteins and lipids, apoptopic changes, and loss of parts of the membrane through vesiculation. One of the major challenges of transfusion medicine is the identification of these alterations and the investigation of whether storage of the blood for a long time may be detrimental to recipients at clinical level;[63] different works have been published dealing with these modifications.[64–67] For doping control purposes, if RBC with alterations due to storage are detected in the blood of an athlete, it may be an indication of the use of blood transfusion. Thus, alterations of RBC due to storage may be used as markers of blood transfusion.

Changes resulting from protein attack by reactive oxygen species, mainly in proteins located in the cytoskeleton were

described in RBC stored for up to 42 days.[64] After seven days of storage, oxidative degradation was observed prevalently in band 4.2, to a minor extent in bands 4.1 and 3, (some proteins are classically named as bands according to their relative mobility in SDS-PAGE electrophoresis)[68] and in spectrin. After 14 days, they were new fragments from β-actin, glyceraldehyde-3-phosphate dehydrogenase, band 4.9, and ankyrin, among others. Another recent work[65] described changes in proteins mainly located in cytoskeleton (affecting spectrin β, band 4.2, ankyrin, tropomodulin, β adductin, band 4.9, tropomyosin) while some changes were also observed in transmembrane proteins (glycophorin C, aquaporin-1, band 3). On the other hand, microRNA profiling of RNA samples from RBC stored at 4 °C for up to 40 days showed that most of microRNA studied (48 out of 52) demonstrated no trend at all; however, four of them showed an increase up to day 20 and a subsequent decrease during storage.[66] To the best of the authors' knowledge, no studies have been published describing the detection of these modifications in blood of individuals subjected to blood transfusion and additional studies are needed to verify the applicability of these approaches to detect the use of blood transfusion in sports.

Another promising hypothesis to find markers of autologous blood transfusion was studied by Pottgiesser *et al.*[69] They analyzed the expression profile of T-lymphocites before and after transfusion, in six volunteers, of autologous RBC concentrates stored for 35 days at 4 °C. After 72 and 96 h post-transfusion, the expression of 728 and 659 genes was altered, respectively, and they were mainly genes coding for proteins that regulate T-lymphocyte activation, adaptative immune response, toll-like receptor pathways, endocytosis of surface receptors, and cell apoptosis. These results showed that the transfusion of autologous blood produced an immune reaction within the T-lymphocytes of the recipient, probably due to the sudden exposure to cell detritus caused by the blood transfusion. However, disturbing factors such as haemolysis or infectious diseases, potentially contributing to false positivity, must be considered when evaluating results.

Conclusion

Blood doping allows increasing and improving of oxygen transport during exercise. Because of the huge advantages that blood doping involves in sport, it is forbidden and highly pursued by WADA.[70] The use of blood transfusions is considered blood doping as it is erythropoietins and other prohibited practices.

The detection of homologous blood transfusions in sport is possible by the use of some tests that use the blood antigens to detect these practices.[8] Nevertheless, there is no methodology currently available in doping control laboratories to detect autologous blood transfusions. Unfortunately, it is well known that the re-infusion of own stored blood is a procedure used as it was reflected a few years ago in the so-called Operation Puerto.[13] Subsequently, an easy screening method which might allow the detection of both types of blood transfusions would be an important breakthrough.

Blood-based parameters are the first logical approach to detecting autologous blood transfusion, in spite of the fact that they do not target the doping agent itself but some indirect markers of their infusion. Of special relevance for the future are the longitudinal changes over time which are planned to be studied by means of the ABP, thus aiming to detect a sudden change due to the blood doping. Also the increase of the haemoglobin mass generated by the transfusion may be of interest in revealing somebody having carried out this practice. The present method using CO rebreathing, however, requires the collaboration of the athlete, which is nearly impossible when dealing with cheating subjects.

Some insights using proteomic and genomic tools are also being suggested for tracing blood transfusion. The former addresses the effect on membrane and cytoskeleton proteins by the storage of RBC. The latter focuses on changes in gene expression profiles after blood re-infusion. Further experiments will clarify the real impact of these new approaches.

More hope is expected from the appearance in the urine of products arising from the bags where the blood is stored, such as the plasticizers approach. The drawback that all subjects excrete some amount of those substances due to common environmental exposure seems to be overturned by the extreme increase in concentrations after a blood transfusion process which offers clear distinction. In case of doubtful results, the combination between plasticizers in urine and blood indirect markers seems a fruitful approach, at least until more selective and valuable detection methods are developed in the future.

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