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Detection of Autologous Blood Transfusions in Athletes: A Historical Perspective

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Autologous blood transfusions (ABTs) has been used by athletes for approximately 4 decades to enhance their performance. Although the method was prohibited by the International Olympic Committee in the mid 1980s, no direct detection method has yet been developed and implemented by the World Anti-Doping Agency (WADA). Several indirect methods have been proposed with the majority relying on changes in erythropoiesis-sensitive blood markers. Compared with the first methods developed in 1987, the sensitivity of subsequent tests has not improved the detection of blood doping. Nevertheless, the use of sophisticated statistical algorithms has assured a higher level of specificity in subsequent detection models, which is a crucial aspect of antidoping testing particularly to avoid "false positives." Today, the testing markers with the best sensitivity/specificity ratio are the Hbmr model (an algorithm based on the total amount of circulating hemoglobin level [hemoglobin level mass] and percentage of reticulocytes, $4.51 \cdot ln(Hbmass) - \sqrt{\%ret}$) and the OFF-hr model (algorithm based on hemoglobin level concentration and percentage of reticulocytes, $Hb(g/L) - 60 \cdot \sqrt{\%ret}$). Only the OFF-hr model is currently approved by WADA. Recently, alternative indirect strategies for detecting blood doping have been proposed. One method is based upon a transfusion-induced immune-response resulting in specific changes in gene expression related to leukocytes such as T lymphocytes. Another method relies on detecting increased plasticizer metabolite levels in the urine caused by the leakage of plasticizers from the blood bags used during the blood storage. These methods need further development and validation across different types of transfusion regimes before they can be implemented. In addition, several research projects have been funded by WADA in recent years and are now under development including "Detection of Autologous Blood Transfusions Using Activated Red Blood Cells (the red blood cells eNOS system)" and "Detection of Autologous Blood Transfusion by Proteomic: Screening to find Unique Biomarkers, Detecting Blood Manipulation from Total Hemoglobin Mass using 15-nitric Oxide as a Tracer Gas, Storage Contamination as a Potential Diagnostic Test for Autologous Blood Transfusion and Test for Blood Transfusion (Autologous/Homologous) based on Changes of Erythrocyte Membrane Protome" (WADA, WADA Funded Research Projects. http://www.wada-ama.org/en/Science-Medicine/Research/Funded-Research-Projects/. 2010). Although strategies to detect autologous blood transfusion have improved, a highly sensitive test to detect small volumes of transfused autologous blood has not yet been implemented.

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BLOOD TRANSFUSIONS, AS a method to enhance endurance performance, first gained attention after the Olympic games (OGs) in Mexico City in 1968. Before these OGs, evidence was presented that a lowered atmospheric pressure would decrease performance in all athletic disciplines dependent on a high level of sustained oxygen uptake [1]. This was indeed confirmed in Mexico City, where all winning times in running races above 800 m were significantly worse than the world records at that time [2]. This highlighted the impact of the oxygen delivery to the working

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muscles as a limiting factor during whole body endurance exercise. It also became evident that runners hailing from higher altitudes tended to be superior to competitors from lowlands because they had "thick blood" with high hemoglobin content. A relatively straightforward way to increase the hemoglobin concentration (Hb), and, hence, oxygen delivery to the muscles is, thus, by blood transfusions. This was documented in the classic study by Ekblom et al [3] from 1972 where a high correlation between Hb and performance capacity after blood withdrawal and reinfusion was presented. An overnight increase in Hb by 13% caused by the reinfusion of 3 units of stored autologous blood resulted in an increase in maximal oxygen uptake and physical performance capacity of 9% and 23%, respectively. The method of transfusing blood in a sport setting was hereafter dubbed "blood doping" by the media, and its potent effect on athletic performance was quickly noted in the sports community. Blood doping was used already at the OG in 1972 by a Finnish steeplechaser, and during subsequent OGs, several athletes admitted having used blood doping [4]. Not until after the OG in Los Angeles in 1984, where the US cycling team used blood doping and won 9 medals after not having won a medal in cycling for 72 years, the method (both homologous and autologous transfusions) was prohibited by the International Olympic Committee, although no method was available to detect its use [5]. A few years later, recombinant human erythropoietin (rhEPO) was developed [6] and marketed. Because of its logistic advantages compared with blood transfusions, rhEPO became the preferred blood boosting method by athletes [7]. At the OG in Sydney in 2000, 2 tests for rhEPO were introduced: a "direct test" that was able to distinguish rhEPO from endogenous molecules by isoelectric focusing [8] and an "indirect test" based on changes in blood parameters caused by rhEPO administration [9]. Because of the introduction of these tests, old-fashioned blood doping reentered the scene. At the Salt Lake City Winter OG in 2002, discarded blood transfusion equipment was found at the headquarters of the Austrian cross-country skiers. After DNA testing, 2 skiers were disqualified [10].Although testing for homologous blood transfusions had been performed at the Lillehammer OG in 1994 by use of antigen testing cards, it was not until 2004 at the OG in Athens that a test had been validated and implemented [11]. At this event, the gold medal winner of the men's time trial in cycling was first tested positive, but because the backup sample (b-sample) was frozen and, thereby, the red blood cells (RBCs) destroyed, no doping offense could be proven. After he failed further doping tests at the 2004 Vuelta a España, the rider was suspended for 2 years [12]. Because of the robustness and long detection window of this test, antidoping experts now consider the use of allogeneic blood transfusions as nonexistent. The obvious alternative to homologous blood transfusions is autologous blood transfusions (ABTs). In 2006, just before the Tour de France, a large Spanish doping scandal evolved, known as Operación Puerto [13]. A doping ring involving several physicians was uncovered by the Spanish police, and more than 200 autologous blood units belonging to professional athletes were found in freezers and refrigerators for subsequent reinfusion [13]. Detailed doping calendars from individual athletes were published, and the modus operandi of involved athletes and their physicians was uncovered [14]. From these calendars, it became evident that besides the massive abuse of a wide range of different performance-enhancing drugs and masking agents, ABTs were used during important competitions. The procedure of blood withdrawal and reinfusion was performed numerous times for each individual athlete during the year by using specialized equipment for phlebotomy and storage. Typically, blood was withdrawn after competitions and reinfused few days before 1-day races or before and during multiple-day competitions. Since then, athletes testing positive for other substances have confirmed the ongoing abuse of ABT today [15,16]. Although decades have passed since the International Olympic Committee prohibited the use of ABT, no method has yet been developed and implemented by the World Anti-Doping Agency (WADA) for its detection.

Over the past several decades, various groups of investigators have explored different methods to detect ABT. In this review, a thorough historical review of the different strategies proposed will be presented, and their limitations and advantages, discussed.

THE USE OF BLOOD MARKERS

The first attempt to develop a test to detect the use of ABT was performed by Berglund et al [17] in 1987. A volume of 1350 mL of blood, corresponding to 3 U of blood, was withdrawn from each subject, stored at 4°C

for 4 weeks, and then reinfused into the donor. The reinfusion of blood resulted in a 60% reduction in serum EPO within the first 24 hours as well as a marked increase in Hb, serum ferritin, and serum bilirubin. From these data, a test based on absolute thresholds of Hb (>12.0 g/dL), bilirubin (>30 μmol/L), ferritin (>33 μmol/L), and EPO (<15 mU/mL) yielded a sensitivity of 50% within the first week of transfusion. Nevertheless, a general problem with using such absolute thresholds is the small within-subject to between-subject variability ratio found for most erythropoiesis-sensitive markers. A conventional reference range, which is usually based on interindividual variability of a defined cohort, will provide a large interval for an individual's values [18]. This interval can be reduced if the blood values from an athlete are compared with his or her historical values [19]. If a new value differs substantially from previous, a sanction could be imposed [19]. Therefore, an alternative "2-sample procedure" was suggested by Berglund et al, where an increase in Hb greater than 5% and a concomitant decrease in serum EPO greater than 50% served as the limits [17]. This algorithm also yielded a sensitivity of 50% but with the window of detection now extending to 2 weeks.

Another important aspect when developing a test for the detection of ABT is the applicability of the test across different transfusion regimens. Historical evidence has shown that athletes and their physicians adjust their doping regimes whenever a new testing technology is introduced [20]. One way of adjusting an ABT regimen is to change the blood storage procedure. Red blood cells undergo structural and biochemical aging-related changes during storage [21], and therefore, the conventional storage of blood at 4°C is allowed for only 5 to 7 weeks depending on storage solution [22]. Because the recovery of reinfused RBCs in the circulation is inversely proportional to their storage period [23], the period of storage at 4°C becomes a compromise between the recovery of reinfused RBCs after reinfusion and the in vivo resynthesis of RBCs during the storage period to compensate for the donated RBCs. An alternative to the 4ºC storage procedure is the freezing of RBCs [24]. Adding glycerol to RBCs minimizes the ice crystal formation in the RBCs during freezing [25,26]. This allows the RBC storage for up to 10 years at −20°C or −80°C [27] and, thereby, sufficient time for a full in vivo recovery of the withdrawn RBCs regardless of the donated volume. Full in vivo recovery is problematic within 7 weeks if more than 1 standard unit of blood (450 mL) is withdrawn [28,29]. However, a drawback with freezing blood is the additional loss of RBCs during the freeze-thaw-wash process [30]. On the other hand, it seems that the postreinfusion survival of the previously frozen RBCs is superior to previously refrigerated RBCs [31]. Therefore, different storage procedures yield different amounts of transfused hemoglobin and hence influence the sensitivity of any new test based on blood markers. Berglund et al [32] used the freezing storage procedure in a follow-up study to validate the sensitivity of his proposed algorithms. The freezing procedure resulted in only modest increases in serum ferritin and serum bilirubin after reinfusion, making the first algorithm unsuitable. The sensitivity of the 2-sample procedure (Hb and serum EPO concentration) was unchanged (50% sensitivity) during the first week after transfusion but decreased to 17% during the second week. The authors cautioned that the specificity (no. of "false positives") of the method had not been evaluated in a larger cohort.

During the 1990s, different sports federations implemented upper hematocrit and Hb thresholds to discourage athletes from manipulating their blood [33,34]. In addition, reticulocyte analysis and thresholds were implemented, but the sensitivity of these thresholds to detect ABT was not examined before 2006. In a study by Damsgaard et al [35], 3 bags of RBCs were reinfused after 4 weeks of refrigerated storage. Multiple samples were collected after withdrawal and reinfusion and analyzed for Hb, hematocrit, reticulocytes count (%ret), serum EPO, and soluble transferrin receptor. The withdrawal of blood resulted in a significant increase in %ret reaching peak levels of 3.0 % 1 week after withdrawal in tandem with a significantly decreased Hb. Withdrawal of blood is the only blood manipulation that results in this specific test result pattern and is therefore highly indicative of ABT. Nevertheless, the collection of samples after withdrawal poses some challenges because RBCs can be stored frozen for years, and therefore, withdrawal can be performed at any time before competition. The time of reinfusion is easier to predict because the optimal performance-enhancing effect is present 1 to 2 days after reinfusion and therefore is likely to be performed close to or in competition [3,36]. After RBC reinfusion, only a modest decrease in %ret from 1.3% to 0.8% was observed.

Individual Hb values were applied to an absolute cutoff threshold of 17.0 g/dL, but only 1 of 10 subjects exceeded this limit. The 99.9% specificity level of the OFF-hr (an algorithm based on Hb and %ret, $Hb(g/L) - 60 \cdot \sqrt{\%ret}$) score developed to detect previous rhEPO administration [37] was not exceeded by any of the subjects. Because all subjects showed variations in Hb of more than 15%, such a change was suggested indicative of ABT. Transfusing smaller volumes (<3 bags) of blood will reduce the degree of the changes observed in Hb and thereby the sensitivity of this method.

Sallet et al [38] presented an alternative 2-sample procedure specifically developed to detect 1 bag of autologous blood. The approach was based on the absolute norms of variations in Hb and OFF-hr score. The blood markers were measured during a period of increased training (21 days), altitude exposure (7 days), withdrawal of 1 bag of blood, a recovery period (19 days), reinfusion of the blood, and a washout period (12 days) [38]. An increase greater than 4% in Hb or 20% in OFF-hr was proposed as thresholds yielding a 100% sensitivity and 100% specificity in the tested subjects. These thresholds were subsequently criticized for the small number of subjects included in the study and the mild type of altitude exposure [39]. The thresholds were validated in a subsequent study, and although the sensitivity was high, 20% and 5% of all measurements from control subjects exceeded the thresholds for Hb and OFF-hr, respectively [40]. The poor specificities for these markers make this model unsuitable for antidoping purposes.

A general problem with using plasma volume–dependent parameters as, that is, the Hb is the increased biologic variability and possibility of masking high values with techniques that produce blood dilution. One way to reduce this effect is to develop algorithms with concentration-derived markers in both the numerator and denominator. An increase/decrease in plasma volume will affect the numerator and denominator equally and cancel the effect on each marker singly. An example of this is the ratio between hemoglobin contained in the mature population of RBCs (RBCHb) and the hemoglobin contained in the reticulocyte population (RetHb) [41]. In a study by our group, we hypothesized that the reinfusion of autologous blood would result in an increased RBCHb ratio and a subsequent decrease in RetHb because of the negative feedback mechanism on erythropoiesis. This effect would be accentuated by the in vitro maturation of reticulocytes during storage and the decreased postreinfusion half-life of stored reticulocytes [42]. Subjects had 3 U of blood withdrawn and were equally divided into a refrigerated (4-week storage) or a frozen (10-week storage) blood storage group. The reinfusion of RBCs resulted in supraphysiologic levels of RBCHb, a consequent decrease in RetHb, and, hence, an increase in RBCHb:RetHb. In addition, in the group that had frozen RBCs reinfused, the %ret in the bags was lower at the time of reinfusion (0.5%) compared with at the time of collection (1.1%). This magnified the increase in the RBCHb:RetHb ratio after reinfusion because the circulating RBC and reticulocyte pools were diluted with RBCs containing a lower RBCHb:RetHb ratio. The RBCHb:RetHb was the most sensitive parameter in detecting ABT after reinfusion when compared with other absolute thresholds for Hb, %ret, and OFF-hr score. The sensitivity was highly dependent on the blood storage procedure. By using a 99% specificity level, 35% and 20% of samples exceeded this threshold during a 4-week period in subjects transfused with frozen and refrigerated blood, respectively. Using a 99.9% specificity level, the sensitivity was reduced to 19% and 4%, respectively.

Hemoglobin Mass

Hemoglobin mass (Hbmass) is another parameter independent of plasma volume shifts. HbMass is the total amount of circulating hemoglobin level in the blood. Because of its lower variability compared with Hb, it has been suggested as a potential candidate for the hematologic module of the Athlete Biological Passport [43]. A biological passport is an approach where certain biologic parameters are measured in an athlete over time. The parameters used should be relatively stable over time (show small biologic variation as well as analytic variation) and change because of doping administration. Relatively large variations are thereby indicative of doping. The hematologic module of the Athlete Biological Passport consists of longitudinal monitoring of Hb and OFF-hr and other RBC indices of blood manipulation that aims to identify suspicious values and profiles [44]. HbMass has been measured for more than a century by the use of carbon monoxide (CO) by the indicator dilution principle [45]. Recently, Schmidt and Prommer [46] modified this method for antidoping purposes by decreasing

the time of CO rebreathing and by making it less invasive. Hemoglobin level mass would be an advantageous parameter in detecting ABT especially during longer periods of strenuous exercise, that is, during the Tour de France, where Hbmass is stable but Hb decreases because of plasma volume expansion [47-49]. Athletes and their physicians are well aware of this physiologic phenomenon and could use it to their advantage. Calendars from Operación Puerto showed that athletes reinfused blood 2 days before the start of Tour de France and then during the tour either on rest days or before major stages to keep the Hb stable. In a noncompetition setting, relatively stable Hb values would be considered normal, but when observed during such strenuous events such as the Tour de France, they must be considered indicative of blood transfusions.

Pottgiesser et al [50] reported the potential of Hbmass to detect autologous transfusion after removing then reinfusing 1 or 2 U of blood in 10 healthy subjects. They found that, after reinfusion of blood, all subjects demonstrated increases in Hbmass beyond the 95% upper reference range (3.3%) associated with the precision (typical error, 2.4%) of the method. Nevertheless, there are some limitations of this study. First of all, the background variation presented by Pottgiesser et al was based on analytic variations only, neglecting any potential biologic variations of Hbmass, making this approach unsuitable when samples are collected more than a few days apart [43]. Second, a 95% reference range would theoretically yield 5% false positives. Such a reference range is commonly used in medical diagnostic testing [51], but in antidoping science, a 99.9% specificity level is adopted by the WADA and used by most sports federations [34,52], assuring a much lower number of false positives. Third, duplicate Hbmass determinations were performed at each measurement point to reduce the analytic error, making the method more time-consuming and impractical in a testing setting. Furthermore, the withdrawn blood was reinfused 2 days after collection, thereby not mimicking a realistic scenario where the resynthesis of hemoglobin level from withdrawal to reinfusion is considered [50]. Because of the inverse relationship between postreinfusion recovery of RBCs and their previous storage period [23], it is likely that the survival of the reinfused RBCs and postreinfusion increase in Hbmass were overestimated compared with during a realistic doping procedure.

A more suitable approach was suggested by the studies of both Prommer et al [43] and Eastwood et al [53], who evaluated the yearly within-subject variations in Hbmass to be 2.2% and 2.1%, respectively. From calculations by Prommer et al, it was proposed that Hbmass assessed by the adaptive model in the Athlete Biological Passport Software (ABPS) Swiss Laboratory for Doping Analyses, Lausanne, Switzerland [44] would yield a sensitivity of approximately 35% at a specificity level of 99.9% when 1 U of blood, corresponding to when 60 g of hemoglobin was reinfused [43]. The WADA recommends in the Operating Guidelines for the Biological Passport that a value or sequence exceeding the 99.9% specificity level should be reviewed by a panel of experts. This implies 1 'false positive' for every thousand analyses. Nevertheless, individual antidoping organizations are allowed to use lower specificity levels before further scrutinization is performed [44]. A bag containing 60 g of hemoglobin level would correspond to an athlete donating 450 mL of blood (standard donation) and having a Hb of 13.3 g/dL, which is a reasonable estimate of the Hb in a well-trained athlete. The 60-g estimate is valid as long as the blood is not buffy-coat reduced or leukodepleted, which will reduce the amount of hemoglobin level by approximately 12 and 5 g, respectively [50]. It is well recognized that the reinfusion of RBCs results in considerable posttransfusion hemolysis of up to 25% within the first 24 hours after reinfusion [54]. Therefore, a sensitivity of 35% would apply only during the first minutes to hours after reinfusion and thereafter decreases as in vivo hemolysis would occur. Using the frozen storage procedure would reduce the amount of reinfused hemoglobin and, thereby, the sensitivity [40]. Therefore, it must be recognized that the volume of the transfused RBC units and the Hb of the donor as well as the processing and storage procedure of the blood all determine the amount of RBCs reinfused and, thereby, the sensitivity of the different blood markers and statistical models.

In another study where 3 U of RBCs were reinfused, the sensitivity of Hbmass was compared with that of Hb, OFF-hr, and a novel algorithm Hbmr, which combines Hbmass with %ret $(4.51 \cdot ln(Hbmass) - \sqrt{\%ret})$ [40]. Hemoglobin mass was the most sensitive parameter to detect short-term RBC transfusions with a sensitivity of approximately 40% during a period of 3 days after

reinfusion, but the sensitivity decreased markedly thereafter. The sensitivity of Hbmass was 0% when 1 U of RBCs was reinfused. The most sensitive marker during the measurement period was the Hbmr: 44% of samples exceeding the 99.9% specificity limit when 3 RBC units were reinfused but 0% when 1 U was reinfused. The most sensitive marker in detection of 1 U of RBCs was the OFF-hr score, with a sensitivity of approximately 12 % during the 4-week period. For all the measured parameters, there was a higher sensitivity in subjects transfused with refrigerated compared with frozen blood. Although the estimated Hbmass has obvious advantages compared with other blood parameters in detecting RBC transfusions, the method has been highly debated in the antidoping community because CO is a potential toxic gas and reduces the exercise capacity for at least some hours after measurement [46]. Furthermore, the measurement is dependent on the collaboration between the tested athlete and the person performing the procedure because the athlete must perform certain breathing patterns to obtain valid measurements. It is therefore highly unlikely that the CO rebreathing procedure will be introduced.

The final study evaluating the potential of blood parameters to detect ABT used a unique setup to replicate the operating procedures of the Athlete Biological Passport as they are today [55]. In the hematologic module of the Athlete Biological Passport, blood results are initially processed by the ABPS developed to identify abnormal blood values or profiles [44]. If a profile exceeds the 99% probability level, the profile is sent to a panel of experts for evaluation. In this study, 21 cyclists were divided into an ABT group or a control group. During a 42-week period, the subjects in the ABT group had 3 to 4 ABTs performed. Three major competitions were scheduled to hypothesize the likely time points of transfusions. One expert was blinded to the subject distribution and transfusion schedule and could schedule up to 10 blood samples from each participant for the ABPS based on knowledge of results from previous tests. His role was to intelligently target subjects for sample collection to reveal deviations in their blood values caused by blood transfusions. During the entire collection period, the OFF-hr model was the most sensitive parameter, identifying 8 (73%) of 11 subjects from the transfusion group at the 99% probability level and 5 (46%) of 11 at the 99.9% probability level. Such high sensitivity is not a result of the short-term changes in the OFF-hr score caused by reinfusion of blood but relies, first and foremost, of an intelligently targeted testing regimen where samples are collected out of competition i.e. after withdrawal of blood, where the OFF-hr score is decreased, as well as in competition when the reinfusion of blood has taken place and where the OFF-hr score is increased. Documents from previous doping scandals (eg, Operación Puerto), where the exact doping administration practices have been uncovered, could be used in intelligently targeting athletes for testing. Additional information from competition schedules, sudden performance improvements, frequent changes in whereabouts information, and trips to remote locations could also be of value.

Plasticizers

A totally different approach to detect ABT was recently proposed by Monfort et al [56]. This approach relies on increased levels of metabolites of di-2-ethyl hexyl phthalate (DEHP) in the urine. The DEHP is a chemical added to plastics such as polyvinyl chloride (PVC) to increase flexibility. The DEHP exists in the PVC matrix as a semisolid and is widely used in medical devices such as blood storage bags [57]. The DEHP molecules migrate out of the PVC matrix into the blood products when they are kept in the blood bags [58]. Considering the largest source of DEHP exposure in humans (ingestion), once DEHP enters the gastrointestinal tract in contaminated foods, it is rapidly hydrolyzed in the small intestine to mono(2-ethylhexyl)phthalate (MEHP) and 2-ethylhexanol via pancreatic lipases [59]. At low concentrations, most of the DEHP is absorbed as its metabolites, although at high doses, a limited amount of unmetabolized DEHP may also be absorbed. Intact DEHP that does cross into the bloodstream is rapidly converted by plasma and liver enzymatic actions to MEHP with a biologic half-life of less than 6 hours [59]. In the study by Monfort et al, the urinary DEHP metabolites, MEHP, mono-(2--ethyl-5-hydroxyhexyl)phthalate, and mono-(2-ethyl-5-oxohexyl)phthalate were measured in 4 different groups: (1) a control group with no known exposure to DEHP, (2) hospitalized patients receiving blood transfusions, (3) nontransfused hospitalized patients receiving other medical care involving plastic materials, and (4) athletes. Samples were collected within a 48-hour window. Urinary concentrations of all 3 DEHP metabolites were significantly higher in patients receiving blood transfusions than in

nontransfused patients and control subjects within the first 24 hours after transfusion.

The method was subsequently validated, and guidelines on the analytic procedure were described in detail [60]. Samples were obtained from (1) 100 healthy volunteers, (2) 10 subjects receiving a blood transfusion, and (3) elite athletes (468 doping control samples). A 99.9% upper reference limit from the presumed nontransfused group of athletes was calculated for 2 of the metabolites. These limits were 4- to 5-fold lower than the lowest concentrations observed in the transfused subjects and could be used to determine abnormal metabolite levels. However, it must be recognized that the data collected are specific for a European population and might specifically reflect the commercial and regulatory trends in the European phthalate market [61,62]. Alternative to absolute thresholds, the method could be implemented into the Athlete Biological Passport as suggested by the authors [56,60]. To do so, the normal variability in urinary DEHP metabolites needs to be determined. This was done in the most recent publication by this group [63]. In this study, 1 bag of blood was drawn from moderately trained subjects and the RBCs reinfused after 14 or 28 days of storage. Longitudinal monitoring of metabolite levels under baseline conditions was performed before transfusions and showed relatively large intraindividual variations in metabolite levels probably due to uncontrolled occasional DEHP exposure. It is therefore doubtful that these parameters can be used in a biological passport before further research into the normal exposure of DEHP has been conducted. When using an universal cutoff limit as suggested by the authors, the window of detection is between 24 and 48 hours after the transfusion.

Another issue is the use of different blood products. Because of differences in solubility of DEHP in various blood products, an RBC unit would deliver more DEHP compared with a whole blood transfusion [64,65]. In the 2 studies, the transfusions are not specified but only referred to as "blood transfusions"[56,60]. In addition, the storage period of the bags would impact the outcome because DEHP in bags increases with storage period [66]. Finally, the window period of detection is of major concern. In the study by Monfort et al [56], the collection intervals after transfusion were divided into samples collected 0 to 24 or 24 to 48 hours after transfusion. The significant increase in metabolite levels for all 3 measured metabolites 0 to 24 hours after transfusion was only evident for 1 metabolite 24 to 48 hours after transfusion. Therefore, the rapid metabolism and clearance of DEHP would seemingly limit sensitivity, and the expected window of detection would be unlikely to exceed 48 hours. Before these different aspects have been addressed, this method can only be used as supporting evidence in, that is, cases of suspicious blood data.

Methods in Development

Although it is unlikely that Hbmass estimation by CO rebreathing will be introduced as a detection method, Hbmass measurement, as such, seems superior to concentration-derived parameters in an antidoping setting not only because the biologic variability is smaller but also because it is unaffected by plasma volume expansion used to mask a high Hb. The WADA has therefore funded research aimed at developing an alternative procedure to measure Hbmass with ^{15}nitric oxide as the tracer gas instead of CO [67]. The general approach will be the same as for Hbmass determined from CO rebreathing, where serial measurements on the same athlete will be performed to reveal unphysiologic deviations. The advantage with the ^{15}nitric oxide is that the amount of inhaled gas can be reduced 4000 times to avoid potential toxic levels in the athletes. Nevertheless, the collaboration of the athlete when performing the measurement is still an issue that must be considered.

Another potential method is the evaluation of changes in the RBCs during blood storage performed by proteomics. The RBC undergoes a wide variety of biochemical and morphological changes during storage, collectively referred to as the "RBC storage lesion" [68]. During prolonged periods of storage, adenosine triphosphate levels decrease, and a sequence of morphological changes occurs. The RBC undergoes crenation and spicule formation to become echinocyte and, subsequently, spheroechinocyte [69]. An additional loss of endogenous RBC antioxidants results in oxidative damage to cytoskeletal proteins [70] and membrane phospholipids [71]. The WADA has funded a project evaluating the proteomic changes in RBCs during blood storage [72]. If these changes can be detected by the proteomic method, the difficult part will probably be to detect them after reinfusion, where they only constitute approximately 4% of the circulating pool of RBCs when 1 U is reinfused (1 U contains ~45 g reinfused into a male endurance athlete with a

Hbmass of 1200 g). In addition, approximately 20% of the transfused RBCs are removed within the first 24 hours. These removal-prone RBCs are the ones with the most severe membrane lesions.

Using changes in gene expression has been suggested as a tool to detect both normal as well as microdoses of rhEPO [73]. Only 1 study has been published, where the applicability of a genomic approach to detect ABT is evaluated [74]. In this pilot study, the authors hypothesized that the storage-induced lesions found in the RBC membrane would expose the detritus of the RBCs to the immune system causing an immune response reflected in genetic changes related to leukocytes such as T lymphocytes. The infusion of 1 U of RBC resulted in significant changes in approximately 700 genes 3 to 4 days after reinfusion. The most significant clusters were formed by genes coding for leukocyte immunoglobulin receptors, Toll-like receptor pathway, genes involved in leukocyte and lymphocyte activation, regulation of endocytosis of surface receptors, adaptive immune response, and cell death/apoptosis. These changes are probably related to processes occurring during RBC storage. Because the study was a pilot study with only 6 subjects, a larger scale study must be performed to validate these findings. In that respect, a control group must be included and measured in a longitudinal fashion to examine the normal variations in the measured genes. It is also of importance to evaluate the potential effects of immune reaction to, that is, infections and hemolysis because the risk of such conditions are increased during prolonged strenuous exercise [74,75]. Another important aspect is the potential effect of changing the storage procedure. In this study, the RBCs were stored at 4°C for 35 days. As storage-induced changes in the RBC membrane increases with the period of storage [23], a reduction in storage period from 5 to 3 weeks might reduce the severity of changes and, hence, the immunologic response after reinfusion. Handling blood for frozen storage reduces the number of RBCs reinfused probably because of the removal of the most apoptosis prone evidenced by a more sustained increase in Hbmass after the frozen storage of RBCs compared with refrigerated stored RBCs (unpublished observations). This will reduce the immunologic response and, thereby, the perturbations in gene expression when previously frozen RBCs are reinfused. Therefore, the effect of a frozen storage procedure as well as a shortening of the refrigerated storage procedure must be evaluated.

In addition, the use of indirect biomarkers such as microRNAs [76] or markers of neocytolosis (selective destruction of immature RBCs during supraphysiologic Hbmass levels) [77] has shown some potential in the detection of rhEPO. These markers are sensitive to changes in erythropoietic activity and might therefore also be applied to ABT.

CONCLUSIONS

Although research aimed to detect ABT was initiated in the mid 1980s, there is still no validated direct testing method that has been implemented by the WADA. In the few studies where appropriate statistical models have been used as indirect evidence through a blood passport approach and reasonable specificities obtained, the most sensitive blood markers have been the Hbmr model and the OFF-hr model. Only the OFF-hr model has, at present, been implemented. Other methods relying on changes in gene expression and increased urinary plasticizer metabolite levels have been proposed. Nevertheless, further studies are needed to determine their sensitivity during alternative ABT procedures. Whether these or alternative methods in development will provide the antidoping community with a sensitive test for the detection of small amounts of transfused autologous RBCs preferably with a long detection window remains to be determined.

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