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Autologous Red Blood Cell Transfusions in Clinics and their Misuse in Sports

Autologe Erythrozytentransfusionen in der Klinik und ihr Missbrauch im Sport

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Summary

- › **The present article** provides an overview on the significance of autologous red blood cell (RBC) transfusion (ABT) in the hospital and its misuse in sports. Such overview seems timely given several ABT doping scandals. ABTs can avoid harms caused by allogeneic blood transfusions (e.g. blood-borne diseases and immunosuppression) and save blood resources.
- › **Still, in clinical practice** ABT has fallen into disuse. Due to the loss of hemoglobin (Hb), blood donation acutely impairs physical fitness. In particular, the loss of iron is critical. It takes 20 days to 2 months for full Hb mass restoration after a ~550 mL phlebotomy ("1-unit"). RBC concentrates are prepared by the removal of plasma and leukocytes, and maintained in plasticized polyvinyl chloride bags.
- › **There are two techniques** for RBC storage: refrigeration (cold storage) or freezing (cryopreservation). In the clinical setting, cold-stored RBCs must be re-infused after 42 days, due to increasing storage lesions, whereas cryopreservation allows for storage for up to 30 years. RBC transfusions can enhance performance. Illegal ABT with cryopreserved RBCs appears to be a method of choice in doping athletes, because no valid laboratory method exists to prove such event. WADA's hematological "Athlete Biological Passport" (ABP) has provided some progress. In addition, "Report Doping" platforms for whistleblowers have been established.

Zusammenfassung

- › **Der vorliegende Artikel** gibt einen Überblick über die Bedeutung der autologen (Blut)-Transfusion (ABT) mit Erythrozytenkonzentrat (EK) in der Klinik und ihren Missbrauch im Sport. Angesichts mehrerer ABT-Dopingskandale erscheint ein solcher Überblick zeitgemäß. Mit autologen EK-Transfusionen können durch allogene Bluttransfusionen verursachte Schäden (z. B. Infektionen und Immunsuppression) vermieden und Blutressourcen eingespart werden.
- › **In der klinischen Praxis** hat die ABT dennoch an Bedeutung verloren. Durch den Verlust von Hämoglobin (Hb) wird die körperliche Fitness durch eine Blutspende initial beeinträchtigt. Insbesondere der Eisenverlust ist kritisch. Nach einer Phlebotomie von ca. 550 mL Blut („1 Einheit“) dauert die vollständige Wiederherstellung der Hb-Masse 20 Tage bis zwei Monate. EK sind von Plasma und Leukozyten befreit und werden in plastifizierten Polyvinylchloridbeuteln aufbewahrt.
- › **Es gibt zwei Techniken** für die EK-Lagerung: Kühlung oder Gefrieren (Kryokonservierung). In der klinischen Praxis müssen gekühlte EK innerhalb von 42 Tagen aufgrund zunehmender Lagerungsläsionen verwendet werden, während die Kryokonservierung eine Lagerdauer von bis zu 30 Jahren ermöglicht. EK-Transfusionen können die körperliche Leistung verbessern. Illegales Eigenblutdoping mit kryokonserviertem EK scheint eine bevorzugte Methode bei dopenden Sportlern zu sein, da es keine valide Labormethode zum Nachweis gibt. Der hämatologische „Biologische Pass“ der WADA hat Fortschritte bewirkt. Darüber hinaus wurden „Report Doping“-Plattformen für Whistleblower eingerichtet.

KEY WORDS:

Anemia, Athlete Biological Passport, Hemoglobin, Doping, Cryopreservation

SCHLÜSSELWÖRTER:

Anämie, Biologischer Pass, Hämoglobin, Doping, Kryokonservierung



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Introduction

Blood or blood products may be transfused into one's own veins ("autologous transfusion") or someone else's veins ("allogeneic" or "homologous"). More than 100 million units of blood are collected globally for this purpose each year (7). Initially transfusions were performed with whole blood, but nowadays specific blood compounds are administered in general. Packed red blood cells (RBCs, erythrocyte concentra-

tes) are infused to treat life-threatening anemia, i.e. when the blood hemoglobin concentration [Hb] has fallen below 70 to 80 g/L in a patient (52). The transfusion of a single RBC unit (from ~525 mL blood) increases [Hb] by about 10 g/L (45).

In endurance sports, the mass of Hb (Hb_{mass}) correlates with the rate of maximal O_2 uptake (O_{2max}). The transfusion of RBCs enhances O_{2max} and

maximal incremental power output, as blood volume and arterial O₂ content increase allowing more O₂ to be transported to the muscles. Therefore, the World Anti-Doping Agency (WADA) has prohibited blood removal and RBC re-infusion in sports, as well as the misuse of erythropoiesis-stimulating agents (23).

The present article provides an overview on the significance of autologous RBC transfusion (ABT) in the hospital and its misuse in sports. Such overview seems timely given several ABT doping scandals, including the recent German-based “Operation Aderlass” (“Operation Bloodletting”). Athletes and their staff should be informed of the fundamentals of blood transfusion.

Physiological Consequences of the Donation of Blood

Standard donors cede about 10% of their RBCs for one unit of blood. Pottgiesser et al. (43) measured Hb_{mass} by CO rebreathing before and after such blood donation (550 mL) in 29 healthy male volunteers. Hb_{mass} was reduced by 75 g (mean value) and recovered after 36 days (range: 20-59 days). These findings are in line with the results of a clinical study of 215 subjects who donated one unit of blood (500 mL) (24). Here, [Hb] declined from 142 g/L to 129 g/L in iron-replete donors (serum ferritin >26 ng/mL) and from 134 g/L to 120 g/L in iron-depleted donors (serum ferritin ≤26 ng/mL). For iron-replete participants not taking iron, the mean time to 80% recovery of [Hb] was 78 days and the median time to baseline ferritin values >168 days. Iron supplementation (ferrous gluconate, 37.5 mg Fe²⁺ orally every day) shortened the mean time to 80% recovery of [Hb] to 31 and 32 days, respectively (24).

A study of 45,000 blood donors has proven that repeated blood donations gnaw on the iron reserves (14). Men subjected to 8-wk inter-donation intervals had a [Hb] of 143 g/L after 2 years (pre-donation [Hb] 150 g/L). Frequent blood donations resulted in symptoms such as tiredness, breathlessness, feeling faint, dizziness and restless legs. Indeed, the loss of iron (200 - 250 mg per 500 mL blood) is critical for blood donors. The physiological day-to-day loss of iron is only 1-2 mg, which is replaced by nutritional iron.

Intestinal iron uptake is inhibited by the hepatic hormone hepcidin, an acute-phase protein. Feedback is given by erythroferrone, a glycoprotein hormone that is produced by erythroblasts and inhibits the synthesis of hepcidin.

Iron deficiency will also develop on apheresis, i.e. the selective sampling of RBCs (two RBC units or more in one time). The iron stores, as indicated by blood ferritin levels, were not replenished to their original levels after 120 days, when 2-unit RBC concentrates were acquired by apheresis (22). The incidence of side effects is not greatly different between whole blood donation and apheresis (50), although patients who undergo apheresis may have faster recovery of postoperative [Hb] and fewer hospitalization days (55).

Due to the loss of Hb, blood donation will negatively affect endurance performance. Ziegler et al. (54) measured [Hb] and iron in blood, O₂max, and time trial (TT) performance before (baseline) and after the donation of one unit blood in 19 healthy men. On day 3, [Hb] was lowered by 7.9%, O₂max by 6.5% and TT performance by 5.2%. [Hb] was not statistically different from baseline 28 days after blood donation (hematocrit was still reduced). O₂max and TT performance were back to baseline 14 days after blood donation. Meurrens et al. (34) measured decreases in maximal power output, O₂max, and Hb_{mass} up to 4 wks after a single blood donation in 24 moderately trained subjects, yielding maximal decreases of 4% (wks 1 and 2),

10% (wk 2), and 7% (day 1), respectively. RBC counts, [Hb], Hct and ferritin values were also lowered and further reduced by the repetition of the blood donations (34).

Features of Autologous RBC Transfusion (ABT)

ABTs can avoid harms caused by allogeneic blood transfusion (e.g. infection and immunosuppression) and save blood resources (reviewed in (55)). Clinically, ABT includes three options: preoperative autologous blood donation (PABD), acute normovolemic hemodilution (ANH, with crystalloid and/or colloid replacement fluid), and perioperative cell salvage (PCS). The knowledge of PABD is relevant concerning blood doping. PABD may be performed in moderately anemic ([Hb] 100 - 130 g/L) patients, scheduled for elective surgery at least 3-5 wks in advance and likely requiring blood transfusion which otherwise cannot be fulfilled. PABD is particularly useful for patients with rare blood types, patients who were transfused with allogeneic blood and produced irregular antibodies, and patients with other blood matching problems (55).

For stimulation of RBC production in the perioperative setting, recombinant human erythropoietin (rhEpo) is licensed in the European Union (EU). There are two approved indications. First, rhEpo (Epoetin alfa or zeta) can be administered to reduce the exposure to allogeneic blood in patients undergoing major elective orthopedic surgery. This therapy intends to augment an already stimulated erythropoiesis at the time of surgery to alleviate postoperative anemia. Second, rhEpo (Epoetin alfa, beta or zeta) can be administered in pre-donation programs to increase the yield of blood in moderately anemic, non-iron deficient, adult patients undergoing major elective surgery accompanied by considerable blood loss requiring pre-deposit of ≥4 units of blood and a high perceived risk for transfusion complications. In this setting, high doses of rhEpo are administered (e.g. 600 IU per kg body weight 1-2 times per wk for 3 to 4 wks prior to surgery). Further, rhEpo is occasionally applied in unapproved clinical situations (“off-label use”), such as in patients undergoing cardiac surgery, and in Jehovah’s witnesses who do not accept blood transfusions (8). RhEpo therapy should be combined with intravenous iron administration.

Regrettably, rhEpo can also be applied in the course of autologous blood doping. Mallorquí et al. (32) demonstrated rhEpo in several bags of blood plasma from elite sportsmen on the occasion of the “Operation Puerto”, an anti-doping investigation in Spain in 2006.

The total transfusion volume prior to elective surgery can be increased by the so-called leap-frog procedure, i.e. the repeated re-infusion of autologous older RBCs and the withdrawal of new RBCs. In an earlier clinical study 300 - 2800 mL (mean 1158 mL) blood was collected during an average of six donations over 14 - 125 days (mean 33 days) (49). Whether the leap-frog technique has been misused for doping purposes is unknown to the author. In clinical practice the leap-frog procedure has fallen into disuse since the risk of hepatitis C virus (HCV) or human immunodeficiency virus (HIV) transmission through allogeneic blood transfusion has been virtually eliminated. In fact, in total only 1,875 units of autologous RBCs were transfused in Germany in 2017 (42), while about 4 millions of allogeneic units are transfused in Germany every year (39). One of the drawbacks of clinical ABT is related to the large portion of pre-donated blood that is not used for PABD but is withdrawn.

Interest in PABD may be roused with the modern “Patient Blood Management” (PMB) concept that favors the use of patients’ own (rather than donors’) blood. PMB involves the

use of multidisciplinary, multimodal, individualized strategies to minimize RBC transfusion with the ultimate goal of improving patients' outcomes (39).

RBC Collection and Refrigeration

Differences in blood collection (manual or apheresis) and in storage methods result in products with different functional characteristics (2). Commonly blood is collected into citrate-dextrose-phosphate solution and RBC concentrates are prepared by the removal of plasma and leukocytes (11, 39). Polyvinyl chloride (PVC) bags plasticized with di(2-ethylhexyl) phthalate (DEHP) are typically used for blood collection and storage (6). The influence of DEHP and two alternative plasticizers, 1,2-cyclohexane-dicarboxylic acid diisononyl ester (DINCH) and n-butyryl-tri-n-hexyl citrate (BTHC), was studied on RBCs stored in PVC bags for 42 days (6). DEHP and DINCH bags offered protection against vesiculation, osmotic stress and loss of Hb, whereas RBCs in BTHC bags stored rather poorly (6). When RBC concentrates in mannitol-adenine-phosphate solution were stored for 6 wks in PVC bags containing DEHP, DINCH and di(2-ethylhexyl) 4-cyclohexene-1,2-dicarboxylate (DOTH), or 4-cyclohexene-1,2-dicarboxylic acid dinonyl ester (DL9TH) and DOTH, there were no significant differences in the total amount of eluted plasticizer, hemolysis and osmotic fragility between the cells in DEHP and non-DEHP blood containers (36).

Non-cryopreserved RBC concentrates are stored at $4 \pm 2^\circ\text{C}$ in a slightly hypertonic additive solution, generally SAGM (sodium, adenine, glucose, mannitol; 376 mOsm/L) (11). Storage in the refrigerator (standard maximum is 42 days) results in RBC "storage lesions". The cells lose their deformability and change their phenotype (e.g. occurrence of spherical cells and echinocytes). As the intraerythrocytic concentrations of adenosine triphosphate (ATP) and 2,3-bisphosphoglycerate (2,3-BPG) decrease, the O_2 affinity increases (these changes are reversible and restored in vivo within a few days). The function of cation pumps is impaired resulting in the loss of potassium and accumulation of sodium. There is irreversible loss of parts of the membrane through vesiculation (11).

Because the collected blood contains RBCs differing in age, the question arises as to the life span of transfused RBCs. Luten et al. (31) studied the recovery of irradiated, leukoreduced RBCs transfused after either short storage (SS, stored 0-10 days) or long storage (LS, stored 25-35 days). The degree of hemolysis and the extracellular bicarbonate, potassium, lactate and lactate dehydrogenase levels of SS RBCs were lower than those of LS RBCs. The mean 24-hr post-transfusion recovery of SS RBCs was 86.4% and that of LS RBCs 73.5%. After the first day, the life spans of the remaining SS and LS RBCs did not differ (116 days vs. 114 days).

Hod et al. (21) studied 14 healthy volunteers who donated two standard leukoreduced RBC units. One unit was re-infused "fresh" (3-7 days of storage), and the other "older" (40-42 days of storage). Contrasting fresh RBCs, re-infusion of the older RBCs caused a mean increase in serum total bilirubin by 0.55 mg/dL at 4 hrs. In addition, transferrin saturation increased progressively over 4 hrs to a mean of 64%, and non-transferrin-bound iron appeared. The higher concentrations of non-transferrin-bound iron correlated with enhanced proliferation in vitro of a pathogenic strain of *Escherichia coli*. Therefore, circulating free iron derived from the rapid clearance of transfused, older stored RBCs may promote bacterial infections. Furthermore, major polyunsaturated fatty acids and their oxidation products (oxylipins) were detected in RBC concentrates stored for 42

days (15). Extracellular vesicles in stored RBC concentrates may cause an inflammatory response in the recipients of older blood (2). Another negative sign is the occurrence of hemolysis or the leakage of Hb from RBCs (19). Regulatory agencies license blood storage systems with a requirement that the rate of hemolysis does not exceed 1% (US Food and Drug Administration) or 0.8% (Council of Europe).

Along these lines, measurements of O_2 max and performance on a cycle ergometer following the re-infusion of autologous RBCs to healthy volunteers have indicated that 42-day RBCs are inferior to 7-day RBCs at delivering O_2 to tissues (5).

Cryopreservation

Therapeutically, cryopreserved RBCs play primary roles in military operations and as a reservoir of RBCs with rare phenotypes (20). RBCs are commonly cryopreserved in relatively strong glycerol solutions at -80°C (3). Scanning electron microscopic investigations of cryopreserved RBCs have yielded increased RBC volumes and shape alterations (41). Although glycerol is a non-toxic substance, its high intracellular concentration and slow rate of osmosis relative to water makes removal of excess glycerol after thawing necessary to prevent osmotic lysis upon transfusion. The process of glycerol removal can be equally damaging to RBCs as the cooling process. Alternative cryoprotectants include betaine (53), trehalose (3), hydroxyethyl starch, and the ice recrystallization inhibitor poly(vinyl alcohol) (13). In contrast to the cooling, the warming process itself is usually benign and can be done rapidly (9). Basically, cryopreservation is technically more difficult than refrigeration. However, a high throughput has been made possible by commercial semi-automated cell washing instruments.

Transfusion of RBCs and Adverse Events

Prior to transfusion, AB0 blood group identity testing must be performed by the physician at the patient's bedside ("bedside testing") (39). Severe immune reactions can occur on mismatched transfusion (1). Whereas for allogeneic transfusions testing of the recipient suffices, for autologous transfusion additionally the packed RBCs must be tested.

The transfusion of RBC concentrates should always be carried out using a standard transfusion set with a filter (pore diameter 170–230 μm). The RBCs should be given via an exclusive venous port; drugs or other agents should not be mixed with the RBCs or administered simultaneously through the same port (39).

Potential risks include transfusion-associated transmissions of bacteria, viruses, parasites or prions and non-immunologically mediated adverse reactions such as transfusion associated circulatory overload (TACO), thrombovascular diseases, hyperkalemia, citrate overload and transfusion hemosiderosis (18). Covin et al. (10) have stated that although the transfusion of autologous RBCs is associated with fewer complications, all of the above untoward events can occur. Namely, the authors have described a case of transfusion-related acute lung injury (TRALI) and hypotension following ABT in a surgical patient (10).

Misuse of RBC Transfusion in Sports

Doping-prone athletes probably turned back to RBC transfusion after reliable detection methods for rhEpo were established (23, 26). Donated cryopreserved RBCs may be stored for up to 30 years, while refrigerated RBCs must be re-infused within

42 days due to increasing storage lesions. Thus, refrigeration is a less probable strategy for ABT doping because it takes at least a month for full [Hb] restoration after blood donation (24, 43, 54). Of note, due to the wash process following cryopreservation, pre-reinfusion handling of cryopreserved blood is associated with a 50% loss of the initially removed Hb compared to only ~30% loss on refrigeration (4). The stored RBCs are probably re-infused to the athletes one to four days before competition (26).

Leigh-Smith (26) has earlier provided a brief review of initial work into the performance-modulating effects of RBC transfusion. Accordingly, the increase in total RBC mass is most important, while the transient increases in blood volume and cardiac output are too short-lived to be of real importance. Leigh-Smith (26) has figured out that a 20 g/L increase in [Hb] would raise the O₂ capacity by 25 mL per liter blood. In an athlete with a mixed venous O₂ saturation of 50% and a cardiac output of 24 L/min, 300 mL extra O₂ would then be available for the tissues per minute (26). In a very recent review, Solheim et al. (48) have evaluated the magnitude of performance enhancement that can be expected from various ABT procedures, as well as the underlying physiological mechanisms. The authors noted that only four of 28 studies were of very high quality, i.e. placebo-controlled, double-blinded crossover studies. In any case, however, RBC re-infusion proved to enhance performance on exercise intensities ranging from ~70 to 100% of O₂max with durations of 5–45 min, an effect also seen in well-trained athletes. A linear relationship has been demonstrated between the volume of donated RBCs and the change in O₂max. RBC re-infusion increases endurance performance by elevating the arterial O₂ content and by reducing lactate concentrations, i.e. reduced anaerobic energy contribution at submaximal intensities (48).

Re-infusion of autologous blood acutely increases blood volume, central venous pressure and cardiac stroke volume (30). However, experimental evidence indicates that the blood volume has usually returned to the pre-transfusion value 24 hrs after re-infusion due to a rapid compensatory reduction in plasma volume (48).

Detection of RBC Transfusion in Athletes

For the detection of allogeneic RBCs, flow cytometry is useful because of the differences in blood group antigens (17, 40). However, there is still no direct method for the detection of re-infused autologous RBCs, despite long-standing research (37, 46, 47).

As an alternative, for chemical detection of ABTs, measurements of metabolites of DEHP in urine have been performed (35). As noted above, however, alternatives to DEHP for RBC storage are available (44), including DINCH, DOTH, and DL9TH.

The WADA has promoted the hematological “Athlete Biological Passport” (ABP), which comprises several blood markers (51). Most significantly, [Hb] and OFF-hr score ($[\text{Hb}] (\text{g L}^{-1}) - 60 \times \sqrt{\text{Ret\%}}$ (percentage of reticulocytes); normal range: 85–95) are considered for sanctioning an athlete. However, there are limitations of the ABP approach with respect to efficacy, sensitivity (rate of detection of correct positives) and specificity (lack of false positives). Mørkeberg et al. (38) re-infused 29 subjects with either one or three units of autologous RBCs. In addition to [Hb] and Ret%, Hb_{mass} was measured one day before and six times after re-infusion. Hb_{mass} proved the only tenable prospect to detect acute transfusions. However, the measurement of Hb_{mass} is based on the inhalation of CO, which is inappropriate in the routine testing for blood doping (46). In a recent study, Malm et al. (33) used multivariate statistics to compare various

blood parameters before and after donation of 450 or 900 mL blood, and until 4 wks after re-infusion of the cryopreserved RBCs. In total, 533 blood samples were included. Over all, a 25% and 86% false positive ratio was achieved in two separate trials. Hematological profiling by multivariate statistics did not reach the WADA stipulated false positive ratio of <0.001% at any time point investigated. A majority of samples remained within limits of normal individual variation at all times (33).

Leuenberger et al. (27) have suggested that the measurement of iron in EDTA-plasma may provide supporting information for the ABP testing. Plasma iron levels increased up to 25-fold 6 hrs after blood re-infusion and remained 10-fold elevated one day after the procedure. A specificity of 100% and a sensitivity of 93% were obtained with a proposed threshold at 45 µg/dL of plasma iron. However, the intake of oral or parenteral iron is a confounding factor for the method. Quantification of hepcidin may be another supportive way to detect ABTs (28). In an experimental trial, healthy subjects received a saline injection for the control phase, after which they donated blood that was re-infused 36 days later (28). Hepcidin concentrations increased 12 hrs and 1 day after blood re-infusion (28). Other investigators have reported Hb profile changes (e.g. reduced HbA1C level) after ABT (25). In addition, altered circulating (29) and erythroid-related (16) microRNAs have been proposed as potential novel biomarkers for detection of ABT misuse in sports. These findings deserve scientific merit, at present. However, the combination of omics-based technologies with classic hematological variables may eventually provide tools for the detection of ABT and other blood doping procedures (46).

Conclusions

ABTs following PABDs in moderately anemic patients scheduled for elective surgery are medically favorable compared to allogeneic blood transfusions (55). The procedure is particularly useful for patients with rare blood group types, irregular antibodies or blood matching problems (55). However, PABD has fallen into disuse in clinical practice, partly due to the large portion of pre-donated blood that is withdrawn.

In contrast, illegal ABT with cryopreserved RBCs appears to be a method of choice in doping athletes, because RBC transfusions can enhance performances, and no valid laboratory method exists to detect autologous RBCs after re-transfusion (33). Previous blood doping scandals are in line with this negative apprehension, including the Spanish-based “Operación Puerto” case in 2006, the German-based “Freiburg University Hospital Case” in 2007, and the German-based “Operation Bloodletting” in 2019. Interestingly, these cases first came to light through admissions by athletes or support personnel. To facilitate whistleblowing, the WADA as well as several National Anti-Doping Organizations (NADOs) and International Federations (IFs) now host ‘Report Doping’ platforms. ■

Conflict of Interest

The authors have no conflict of interest.

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