Ex vivo erythrocyte generation and blood doping

Giovanni Lombardi[1], Giuseppe Banfi[1,2], Giuseppe Lippi[3], Fabian Sanchis-Gomar[4]

[1]I.R.C.C.S. Galeazzi Orthopaedic Institute, Milan; [2]Chair of Clinical Biochemistry, School of Medicine, University of Milan, Milan; [3]Unit of Diagnostic Blood Biochemistry, Department of Pathology and Laboratory Medicine, University Hospital of Parma, Parma, Italy; [4]Department of Physiology, Faculty of Medicine, University of Valencia, Fundacion Investigacion Hospital Clinico Universitario/INCLIVA, Valencia, Spain

Doping in sport is widely used by both professional and non-professional athletes to improve performance and increase the probability of success. Anabolic substances are mainly used by dishonest athletes practising power sport disciplines, whereas blood doping is mostly used by those who perform endurance and aerobic sports[1,2]. Blood doping is based on the use and administration of any illicit substance or procedure aimed at increasing and optimising oxygen delivery to the exercising muscles and, therefore, includes blood transfusions, administration of erythropoiesis-stimulating substances (namely hormones and growth factors), blood substitutes and artificial altitude facilities, possibly mixed and/or combined[3,4].

The use of blood transfusions, either autologous or homologous, is an effective strategy for increasing oxygen delivery to the muscles and can be used immediately before a single competition or even during long-lasting sporting events to maintain a stable haemoglobin (Hb) concentration, especially when a natural decrease is expected as a result of physiological adaptation to endurance exercise. The procedure is not new, since it became popular nearly 40 years ago, but then suddenly declined due to its inclusion among the list of prohibited methods issued by the International Olympic Committee (IOC). Another reason that contributed greatly to the sudden decay of popularity of blood transfusions among elite athletes was the cloning of the erythropoietin gene and subsequent development of recombinant erythropoietin (rHuEpo) and its introduction among the armamentarium of illicit ergogenic aids. The great success of rHuEpo and analogues (erythropoiesis-stimulating agents, ESA) was mainly the consequence of the remarkable biological and technical advantages that these substances had over traditional means of increasing the red cell mass, such as blood transfusions. These advantages include easier supply (from healthcare facilities as well as from the "black market"), more comfortable administration (small subcutaneous doses) and the sharp and long-lasting effects on erythropoiesis (i.e. "blood boosting"). Once the concentration of Hb has been raised through ESA administration, the high concentration can be maintained by weekly administered microdoses of ESA which have a detection window of only 12-18 hours, for rHuEpo, compared to 3 days for regular doses[5,6].

The subsequent development and implementation of (more or less) reliable electrophoretic techniques to screen for rHuEpo misuse caused a sudden resurgence of blood transfusions, which also took advantage of new procedures for collecting and storing the erythrocytes (e.g. freezing), allowing their use over a very long period of time as well as their harvesting during rest periods, thus avoiding a decline of Hb and aerobic sport performance during the competitive season[7].

The predominant form of blood doping by transfusion is autologous blood transfusion, i.e. the blood donor and the recipient are the same. Homologous transfusions, in which the blood is drawn from a donor who is not the recipient, can also be used, but the potential side effects and the easier identification by traditional red blood cell (RBC) phenotyping and flow cytometry substantially limit the use of this practice[7,8].

The detection of autologous blood transfusion is more challenging. A direct method based on the identification of plasticizers excreted in the urine as a result of autologous blood transfusion was recently proposed as a reliable approach to detect such transfusions in athletes, although it has shown several drawbacks and is not currently implemented as a doping detection tool[9-11]. However, an indirect approach, based on the assessment of Hb concentration and reticulocyte percentage (Ret%) over time, can be used and also combined in the so-called OFF-score (i.e. Hb-60√Ret%)[5]. These parameters are introduced into a statistical programme (i.e. the Athlete's Biological Passport), which is aimed to unmask non-physiological perturbations of blood homeostasis such as those following a blood transfusion (abnormal Hb increase and a decrease in Ret%) or withdrawal of blood for the purpose of storage and subsequent re-administration (abnormal Hb decrease combined with an increase in Ret%)[12]. Absolute values of Hb and Ret% along with other derived parameters (mean corpuscular volume, Ret%, Hb, etc.) might also be used for screening for blood transfusions, but the results provided so far have been rather controversial[13,14],

despite the fact that the high sensitivity of the passport approach for detecting blood transfusion has been unequivocally proven in a blinded experiment in healthy subjects[15]. Although the passport approach is useful in the case of "regular" amounts of re-infused blood (3 units), its low sensitivity in detecting smaller amounts (1 unit) still represents a major limitation[6]. The leading issue in detecting autologous blood transfusion is the potential dilution of intravenously administered RBC and their early, partial removal[6].

It should be mentioned that the organisation and management of the collection and long-term storage of blood bags is difficult, especially outside healthcare facilities, so that this practice is mostly limited to high-profile, wealthy athletes.

The widespread availability and relatively low cost of blood for transfusions are paramount needs for modern healthcare systems. Blood requirements for surgical and medical treatment are increasing steadily while the availability is stable or even decreasing, so that the demand is already exceeding the supply. Current technology and increasing knowledge on tissue and cell engineering, especially those based on the use of haematological stem cells to obtain mature and efficient blood particles, is opening appealing strategies and approaches for obtaining safe and disposable blood to be used for clinical purposes. RBC have been widely investigated in the past, and their metabolic pathways and mechanical properties have been exhaustively described and also reproduced *in vitro*[16,17]. The production of biomaterial particles with similar (or even identical) chemical and physical properties as those of mature, functional RBC has been reported[18], so we have already alerted the scientific community as well as anti-doping authorities on the possible, surreptitious use of these bioengineering techniques for unfair and illicit purposes in sports[19].

The generation of RBC from haematopoietic cells or human induced pluripotent stem cells can now be considered as realistic[20-23] and can be accomplished on an industrial basis. Understandably this is a most favourable achievement for healthcare systems and patients in order to face the increasing demand for a wider availability of blood[24], but is also a serious threat for sport medicine, since it reasonably offers the possibility of effective blood doping. The techniques that have been described so far can be used to obtain very large quantities of RBC, yielding as much as a 130-fold increase over the original quantity of progenitor cells and, thereby, producing nearly 560 units of RBC per umbilical cord donation (assuming 5×10^6 CD34+ cells per donation)[25]. Umbilical cord blood is an optimal source for medical and ethical reasons, although it is also viable for blood doping, since the RBC that can be obtained are safe and highly controlled after selection of proper antigenic determinants. The procedure can even be implemented using bone marrow-derived hematopoietic cells or fibroblasts from the recipient. However, more realistically, the use of these biotechnological approaches in the doping context could become plausible only after the development of methods based on the use of hematopoietic stem cells derived from peripheral blood.

The manufacture of RBC could overcome several problems of the procedures currently used in the doping context for blood collection and storage. Long storage is not required and the production can be tailored according to the athlete's needs. Moreover, the RBC are fresh and thereby more functional with regards to gas exchange than those maintained in blood bags, even when properly stored. The amount of RBC administered intravenously can be regulated by appropriate dilution to avoid anomalous laboratory test results even with proteomic studies[6] and the biological characteristics and behaviour of these "new" RBC do not differ from those of their "natural" counterparts. To the best of our knowledge, the only indices that could be used in the anti-doping context are shape and dimensions, which seem to be slightly greater for manufactured RBC[25].

In conclusion, the scientific as well as the sport medicine communities should be aware of the risk that these novel approaches to the generation of RBC could have with regards to blood doping and should cooperate closely to increase knowledge about RBC manufacturing and to develop appropriate tests to identify their unfair use in sports.

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Correspondence: Giovanni Lombardi
Laboratory of Experimental Biochemistry and Molecular Biology
I.R.C.C.S. Istituto Ortopedico Galeazzi
Via R. Galeazzi 4
20161 Milan, Italy
e-mail: giovanni.lombardi@grupposandonato.it