DETECTION OF BLOOD DOPING IN ATHLETES (MASKING SUBSTANCES AND METHODS)

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Abstract

The evidence in the detection of blood doping among endurance athletes is conflicting especially when these athletes are undergoing autologous blood transfusions and abusing recombinant human erythropoietin (rHuEPO). Continuous monitoring all year round and creating haematological profiles, known as athlete's haematological or blood passport with cut-off values, are being practiced by some international sport federations to ban athletes from participating or apply sanctions and punishments when the tested athletes are proven to be doped. However, athletes who use prohibited substances or methods that increase the concentration of red blood cells and haemoglobin (Hb) are also using masking agents and other methods to manipulate the samples. The use of plasma expanders are prohibited as they induce haemodilution and can potentially result in false negatives of athletes' blood samples. Other substances to date, specifically the ingestion of Creatine (Cr) and Glycerol (Gly), can cause plasma expansion when taken together by their novel synergistic effect on the human body and particularly on total body water (TBW). However, Gly has recently been added to the WADA 2010 prohibited substances list (following the completion and publication of some of the results from the experiments presented here). In addition, the posture of the sampled athlete during blood collection may also influence the concentration of blood parameters and potentially plasma volume. The main objectives of the studies presented in this thesis are to test the hypothesis that the use of hyperhydration agents, particularly Cr and Gly, in conjunction with postural changes will dilute blood samples to the extent that this manipulation could potentially influence the results of a blood doping test.

In the first experiment (EXP1), eight subjects volunteered to take part in Cr and Gly fluid loading for 7 days. Urine samples and body mass were obtained before subjects lay in a supine (0°) postural position for 30 min. During that period, body water compartments were estimated and urine and blood samples were obtained from the subjects. This hyperhydration strategy and posture resulted in a significant increase in body mass by 0.82 ± 0.22 kg, TBW by 0.67 ± 0.17 L, intracellular water (ICW) by 0.35 ± 0.09 L, extracellular water (ECW) by 0.37 ±

0.15 L and urine osmolality by 161 ± 115 mOsmol.kg^{-1} ($P<0.05$) with significant decreases in Haematocrit (Hct) values at 10 min, 20 min and 30 min blood samples by 1.26% ± 0.65%, 1.3% ± 0.96% , 1.35% ± 0.73%, respectively, ($P<0.05$) and an insignificant decease in Hb concentration ($P=0.07$). These results showed that the fluid loading protocol and posture used induced a degree of hyperhydration and haemodilution that could potentially influence the outcome of a drug test.

The second experiment (EXP2) focused on the changes in Hb and Hct with different postural positions of ten euhydrated subjects. The extensive research done to detect blood doping by indirect markers and equations used the supine postural position for 5-10 min prior to obtaining blood samples. However, WADA's only approved postural position is to have the selected athlete, undergoing a doping test, be seated for 10 min before venipuncture. However, most of WADA's anti-doping research relating to this application have been based on tests developed with subjects tested in a supine position. In this experiment, three postural positions were applied: Supine (0°), sitting and standing. As subjects changed their postures from supine (0°), sitting and ending with standing, there was an increased haemoconcentration as reflected by an increase in Hb and Hct values ($P<0.05$). Hb concentration and Hct values increased by 0.55 ± 0.06 g.L^{-1} and 0.87% ± 0.12%, respectively, when subjects changed their posture from supine to sitting and increased by 0.71 ± 0.14 g.L^{-1} and 1.70% ± 0.08%, respectively, when subjects changed their posture from sitting to standing. This experiment showed that the posture in which the blood samples were obtained from human subjects has a direct effect on the concentration of blood parameters such as Hb and Hct. While not a new finding, this observation confirmed the idea that posture requires standardisation for meaningful research outcomes aimed at developing valid doping tests to be developed (currently not the case).

In the third experiment (EXP3), seven healthy trained subjects volunteered to take part in Cr and Gly hyperhydration followed by 2 running bouts at 60% of their maximum oxygen uptake (VO_2 max) in an environmental chamber. This experiment employed a different Cr and Gly hyperhydration strategy to the one used in EXP1. Specifically, Cr was loaded for 7 days and Gly was only ingested on

the day of experiment and precisely 5 hours prior to the first blood sample. Each exercise bout consisted of 30 min running bouts but at two different climatic temperatures 10°C and 35°C, respectively with 70% relative humidity. Body mass and urine samples were taken before the start of the test. Blood samples were obtained after the subjects were comfortably seated for 10 min and just prior to the start of the test as well as after each running bout. This protocol resulted in no significant changes in Hb, Hct, Reticulocytes (Retics%) and OFF-hr scores and values. In general this revised hyperhydration regiment did not induce the same degree of haemodilution and plasma expansion as the previous approach and could therefore account for the negative findings on blood profiles.

The hyperhydration protocol of EXP1 using Cr and Gly for 7 days succeeded in significantly manipulating and increasing body mass, TBW, ICW, ECW and urine osmolality ($P<0.05$) and decreasing Hct ($P<0.05$) values but not Hb concentration ($P=0.07$) when blood was withdrawn in supine (0°) posture. In EXP2, the change of posture from supine (0°), to sitting and ending with standing resulted in haemoconcentration and increases in Hct and Hb values. With a slight change of hyperhydration protocol in EXP3 of Cr for 7 days and Gly on the seventh day only, it resulted in no significant changes in Hct, Hb, %Retics and OFF-hr scores and therefore no haemodilution effects when compared to pre-supplementation tests at rest and following submaximal exercise bouts in cool and warm environments. In all EXP1, EXP2 and EXP3, Hct among other blood parameters was the most affected by hyperhydration, posture and time at which blood samples were obtained. The hyperhydration protocol of EXP1 was more successful in decreasing Hct and Hb values than EXP3.

More research should be focused on determining whether the extent of blood dilution induced by hyperhydration strategies such as those used in the present series of experiments can alter the outcome of a doping test. Other hyperhydration/fluid loading protocols should be assessed including plasma expanding substances and methods in view of these substances being considered by WADA to be included in the prohibited substances and methods list (if proven effective). It is recommended that the emphasis of WADA-related research is also shifted to the identification of other physiological/molecular markers that are not significantly affected by haemodilution such genetic signatures in

response to drug perturbations (e.g. autologous blood transfusions and rHuEPO injections).

Key words: Doping, Blood Doping, erythropoietin (EPO), erythropoiesis, recombinant human erythropoietin (rHuEPO), urine tests, blood transfusions, blood tests, haemoglobin (Hb), haematocrit (Hct), reticulocytes (Retics%), OFF-hr score, plasma expansion, hyperhydration, haemodilution, Creatine (Cr), Glycerol (Gly).

Table of Contents

List of Tables

List of Figures

List of abbreviations

%Retics	Reticulocytes Haematocrit
°	Degree (s)
°C	Centigrade Cilices degree (s)
2,3-DPG	2,3-diphosphoglycerate
ADO	Administrative Doping Officer
ADP	Adenosine diphosphate
ASL	Above sea level
ATP	Adenosine triphosphate
CERA	Continuous erythropoietin receptor activator
cm	Centimetre
CO_2	Carbon dioxide
Cr	Creatine
DCO	Doping Control Officer
ECW	Extracellular water
EPO	Erythropoietin
EXP1	Creatine and Glycerol Hyperhydration Experiment
EXP2	Postural Changes Experiment
EXP3	Running with Creatine and Glycerol Hyperhydration Experiment
FIS	International Ski Federation
g	Gram
$g.day^{-1}$	Gram per day
$g.L^{-1}$	Gram per Litre
$g \cdot kg^{-1}$	Gram of per kilogram of body mass
Gly	Glycerol
h	Hour(s)
Hb	Haemoglobin
HBOCs	Haemoglobin Based Oxygen Carriers
Hct	Haematocrit
HIF	Hypoxia-inducible factor
HR	Heart rate
HRE	Hypoxia regulatory element

IAAF	International Associations of Athletics Federations
ICW	Intracellular water
IOC	International Olympic Committee
$IU.kg^{-1}$	International Unit per kilogram
kg	Kilogram
$kg\cdot(m^2)^{-1}$	Kilogram per meter square
L	Litre
m	Meter(s)
min	Minute(s)
$ml.h^{-1}.kg^{-1}$	Millilitre per hour per kilogram
$ml.L^{-1}$	Millilitre per litre
$ml\cdot kg^{-1}.min^{-1}$	Millilitre per minute per kilogram
mm Hg	Millie meter Mercury
$mmol.L^{-1}$	Millie mole per Litre
$mOsmol.kg^{-1}$	Millie osmole per kilogram
NADO	National Anti-Doping Organization
NESP	Novel Erythropoiesis-Stimulating Protein
O_2	Oxygen
OFF-hr	2nd generation blood doping detection model (EPO off cycle)
PCn	Phosphocreatine; phosphorylated creatine
PCV	Packed cell volume; Hct
PFCs	Perfluorocarbon Emulsions
pO_2	Oxygen partial pressure
RADO	Regional Anti-Doping Organization
RBCs	Eryhrocytes; red blood cells
Retics	Reticulocytes
rHuEPO	Recombinant human erythropoietin
s	Second(s)
sTfr	Soluble transferrin receptor
TBW	Total body water
UCI	International Cycling Union
VO_2 max	Maximum oxygen uptake
WADA	World Anti-Doping Agency

“Pressure is good because you can not work without pressure.”
(*a wise person*)

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Author's Declaration

I herby declare that this thesis has been composed by myself, and the work of which it is a record has been done by myself, except where specifically acknowledged. I also confirm that it has not been submitted in any previous applications for higher degrees and that the sources of information have been specifically acknowledged by means of references.

Some of the results contained in this thesis have been published in a peer-reviewed Journal as follows:

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Signed ……..

Date ……..

CHAPTER 1

GENERAL INTRODUCTION

1. General Introduction

1.1. Doping Definition

According to the International Olympic Committee (IOC), Doping is defined as ***"the use of physiological substances in abnormal amounts and with abnormal methods, with the exclusive aim of attaining an artificial and unfair increase of performance in competition"*** (Dugal and Bertrand, 1976; as cited by Wilber, 2002). However, after the 2000 Olympic Games, The World Anti-Doping Agency (WADA) established the World Anti-Doping Code 2003 and added some other specifications to the word "Doping" rather than the attempt to use a prohibited substance or a prohibited method. WADA considers Doping as the violation of the World Anti-Doping Code rules by the incidence of one or more than one aspect; ***"Doping is defined as the occurrence of one or more of the anti-doping rule violations set forth in Article 2.1 through Article 2.8 of the Code"***. (The World Anti-Doping Code 2009, WADA, 2009b). In relation to these rules, WADA can consider whether that any tested athlete is clean or doped, guilty or not guilty, and/or a cheater or a champion. ***"The World Anti-Doping Code was first adopted in 2003 and became effective in 2004. The enclosed incorporates revisions to the World Anti-Doping Code that were approved by the World Anti-Doping Agency Foundation Board on November 17, 2007. The revised World Anti-Doping Code is effective as of January 1, 2009"*** (The World Anti-Doping Code 2009, WADA, 2009b).

1.2. Blood Doping Definition and Reasons

Blood doping is the application of methods and use of substances for non-medical reasons by healthy athletes to enhance oxygen (O_2) delivery to tissues to increase their maximal aerobic power leading to improved aerobic athletic performance (Gaudard et al., 2003; Lippi et al., 2006; Elliott, 2008). The artificial manipulation of the endurance performance enhancement either by using a prohibited substance or by the use of any prohibited method to enhance O_2 transport to skeletal muscles and is therefore considered "doping" and when directly related to the blood content and parameters is specifically called "blood doping". In addition, injecting the hormone erythropoietin (EPO), which is a

prohibited substance, and EPO gene manipulation are considered “blood doping” (The 2009 Prohibited List International standards, WADA, 2009a). The Injection of artificial blood substitutes, red blood cells (RBCs) or whole blood of any origin whether by autologous, homologous or heterologous transfusions, which are prohibited methods, are also considered “blood doping” (The World Anti-Doping Code 2009, WADA, 2009b; The 2009 Prohibited List International standards, WADA, 2009a).

The aerobic endurance performance can be assessed by the delayed time to exhaustion and muscle fatigue and the increased maximum oxygen uptake (VO_2 max), defined as the highest rate at which O_2 can be taken up and used by the body and skeletal muscle tissues during severe exercise (Gaudard et al., 2003). The increase in Hb concentration in blood, particularly in red blood cells (RBCs), and the manipulation of Hb-O_2 affinity and release (naturally and artificially induced shifting of the Hb-O_2 dissociation curve to the right) increase the efficiency of O_2 transport and delivery to peripheral and muscular tissues, improves and increases the energy production leading to increased muscular fatigue resistance and finally a better athletic performance (Lippi et al., 2006; Gaudard et al., 2003; Elliott, 2008). The latter outcome leaded to legal (healthy) and illegal (doping) practices of methods and substances mainly in endurance athletic competitions.

1.3. Prohibited methods and substances used for Blood Doping and Enhancement of O_2 delivery Capacity

The increase in O_2 delivery to skeletal muscle tissue can be manipulated and achieved by direct and indirect actions on Hb (Gaudard et al., 2003). See figure 1.1 (Adopted from Gaudard et al., 2003, Lippi et al., 2006 Pascual et al., 2004 and Elliott, 2008).

Figure 1.1 Prohibited Methods and Substances of Blood Doping (Adopted from Gaudard et al., 2003, Lippi et al., 2006, Pascual et al., 2004 and Elliott, 2008).

1.3.1. O_2 Delivery Enhancement by increasing Hb Mass and Concentration in Blood

1.3.1.1. Blood Transfusions

The infusion of whole blood or packed RBCs into human body intravenously is called "blood transfusion" and can be classified into 3 different categories according to the source of donation: heterologous, homologous and autologous transfusions. As mentioned earlier, all of these methods are prohibited to be used by athletes according to WADA's rules and regulations (The World anti-doping code, WADA, 2009b; The 2009 Prohibited List International standards, WADA, 2009a).

Heterologous transfusions are transfusions of a specie's blood to another specie; animal's blood (e.g. calf and turtle's blood) into human body or vice versa for clinical and research reasons and to human and animal athletes in sports (Parisotto, 2006). Homologous blood transfusion is the infusion of whole or part of blood from the same specie but not from the same person; i.e. the transfusion of another person's blood (Parisotto, 2006). On the other hand, autologous blood transfusion is the infusion of whole or part of a person's own blood (Parisotto, 2006) whereas the blood donor is the same as the transfusion recipient (Lippi et al., 2006). The basic use of blood transfusions is to save and enhance the life of patients suffering critically from acute and chronic anaemia and because patients will not usually require the whole blood contents, the whole blood can be separated into components with the advanced biotechnology and the infusion of that component would be according to the patient's specific diagnosis and prognosis (Lippi et al., 2006). Autologous blood transfusion starts with the withdrawal of 1 to 4 blood units, in which 1 blood unit consists of 450 ml of whole blood or 225 ml of concentrated RBCs, several weeks prior to competitive events (Lippi and Banfi, 2006) followed by the reinfusion of the stored blood units several days prior to any competitive event (Ashenden, 2002). The withdrawn amount of blood is immediately replaced by the infusion of isotonic saline solution, then the blood is centrifuged and RBCs are separated and stored as units either by refrigeration at +4°C or frozen at -80°C and finally these

stored RBC units are heated to +41°C prior to reinfusion (Morkeberg et al., 2009a).

Blood transfusions can elevate individual's Hb concentration above normal that consequently increases VO_2 max (Ekblom, 2000). This explains the success of USA's cycling team in the 1984 Los Angeles Olympic Games with the obvious abuse of blood transfusions (Shaskey and Green, 2000). The reinfusion of 1350 ml of autologous blood in six elite cross-country skiers were compared to control group of seven subjects resulted in an increase of Hb by 7.9% from before the phlebotomy sample and 14% before the reinfusion sample (Berglund et al., 1987). In a follow up study, The autologous transfusion of 1350 ml of 6 well trained cross country skiers resulted in improvements in race performance times after 3 hours and 14 days of reinfusion (Berglund and Hemmingson, 1987). The reinfusion of 1350 ml of freezer stored autologous blood in 12 well trained male and female former endurance athletes, phlebotomised 3 to 4 months prior to reinfusion, resulted in increased Hb concentrations significantly (Berglund et al., 1989).

Blood transfusions are not risk free methods of doping and can be fatal although it can lead to increases up to 20% of Hb and Hct (Lippi et al., 2006). Hct is the percentage of blood made up by RBCs (Parisotto, 2006) and sometimes called packed cell volume or erythrocyte volume fraction (Thirup, 2003). The main complication of blood transfusions is the hyperviscosity syndrome characterised by the elevated blood thickness and viscosity, lowered cardiac output, decreased blood flow velocity that eventually results in reduced O_2 delivery to peripheral tissues (Lippi et al., 2006). Transfused homologous blood induces reactions like: urticaria, fever and anaphylactic shock, blood born infections like: HIV, syphilis, hepatitis, cytomegalovirus, malaria and Creutzfeldt-Jakob disease (Lippi and Banfi, 2006). Autologous transfusion is safer, more convenient and maybe cheaper from homologous transfusion (Corrigan, 2002).

1.3.2. Stimulation of Endogenous EPO and Erythropoiesis

Erythropoietin (EPO) is a glycoprotein hormone, which is mainly produced and secreted by the by the kidneys (Jacobson et al., 1957) and maybe up to 10% is

made in the liver (Fried, 1972; Fisher, 1979). EPO stimulates the proliferation and differentiation of erythroid progenitor cells in the red bone marrow to develop into erythrocytes and then released into the blood stream (Gaudard et al., 2003). The newly released erythrocytes are immature RBCs containing nuclei and called reticulocytes (Retics) that contain residues of nucleic acid RNA (Banfi, 2008). Retics have a larger volume than RBCs with identical Hb content but lower Hb concentration (D'Onofrio et al., 1995). RBCs have an average lifespan of 120 days however Retics are present and stay in the circulating blood for a period of 1-4 days until they loose their nuclei and full maturation to RBCs is achieved (Zucker-Franklin and Grossi, 2003).

1.3.2.1. Altitude Training and Simulated Altitude Exposures

High altitude causes tissue hypoxia and organisms respond and adapt to this condition by erythropoiesis (Samaja, 2001). Hypoxia causes transcriptional activation of various genes for the encoding of glycolytic enzymes and growth factors and peptides, like EPO, which are regulated and controlled by hypoxia-inducible factor 1 compound (HIF-1) that is responsible for the regulation and transcription of genes with hypoxia regulatory element (Gaudard et al., 2003; Mounier et al., 2006; Mounier et al., 2009). The term "adaptation" refers to the physiological adjustments and modification that took place over generations under stable and continuous exposure to high altitude whereas the term "acclimatisation" describes the physiological changes of short exposures to high altitude (Lippi et al., 2006). According to Bartsch and Saltin (Bartsch and Saltin, 2008) the elevation and ascending above sea level (ASL) are classified into the following: sea level - 500m ASL as a "near sea level", 500m – 2000m ASL as a "low altitude", 2000m – 3000m ASL as a "moderate altitude", 3000m – 5000m ASL as a "high altitude" and any altitude above 5500m ASL as an "extreme altitude".

Altitude and hypoxic training can be classified into 3 approaches in which athletes live and train at high altitude, live at low altitude but train at high altitude, live at high altitude and train at low altitude (Wilber, 2007). In 1991, levine et al. designed the first study to be conducted with the concept of "living high and training low" (Levine et al., 1991) and was shown to be the most

effective approach to improve athletic sea level endurance performance (Levine and Stray-Gundersen, 2001). Altitude and hypoxic training can be achieved by the elevation to natural terrestrial altitudes, simulated by hypoxic devices or mixture of both natural and simulated high and low altitudes (Wilber, 2001; Wilber, 2007; Bartsch and Saltin, 2008). Simulated hypoxia and normoxia can be acquired by nitrogen dilution, supplemental O_2, intermittent hypoxic exposures, hypoxic apartments and sleeping devices such as Colorado Altitude Training Hatch® and Hypoxico Tent System® (Wilber, 2001). The brief exposure to hypoxia of 1.5-2.0 hours using theses devices hypothetically sufficient to stimulate RBCs synthesis (Wilber, 2001).

Acclimatisation of four weeks at high altitude in conjunction with high intensity training at low altitude was shown to sufficiently enhance the athletic performance at sea level in female and male endurance elite athletes (Stray-Gundersen et al., 2001). Twenty four days of living high at 2500m ASL and training low at 1000m and 1800m ASL resulted in increased erythropoiesis (serum EPO, transferrin, sTfr, Retics, Hb mass and RBC volume), increased VO_2 max and improved 5000m performance times in elite male and female endurance skiers (Wehrlin et al., 2006). Living at simulated altitude of 2650m ASL and training at 600m ASL for 5 days was sufficient to increase serum EPO levels significantly but did not increase Retics production in 6 elite male runners (Ashenden et al., 2000). Altitude acclimatisation and training effects on athletic performance has been shown in a study to have interindividual variability described by "responders" and "non-responders" to altitude training although the plasma EPO concentration and RBC volume was significantly raised in all subjects but the significant improvements in VO_2 max and endurance performance were only observed in the "responders" (Levine and Stray-Gundersen, 1997). These findings where confirmed in a follow up study of living high and training low concept that there are individual variations in athletes responding to altitude training (Chapman et al., 1998). HIF-1, which controls the expression of EPO gene upon exposure to hypoxia, can not be used to identify the individual variations among "responders" and "non-responders" to live high and train low concept (Mounier et al., 2006). Numerous studies recruiting athletes for altitude and hypoxic training have shown significant increases in serum EPO concentrations without improving performance, VO_2 max or any erythropoietic

responses of increasing Hct, RBC volume, Hb, Retics or soluble transferrin receptor (sTfr) (Ashenden et al., 2000; Julian et al., 2004; Robach et al., 2006; Abellan et al., 2007; Calbet et al., 2002; Ventura et al., 2003; Dehnert et al., 2002; Abellan et al., 2005).

Training at high altitude or in hypoxic environments reduces athletes' performance speed, power output and VO_2 max (Levine, 2002). The acclimatisation to high altitudes leads to suppression of appetite, protein synthesis inhibition, mild muscle atrophy and compensatory metabolic responses (Levine and Stray-Gundersen, 2001). With further elevation to higher altitudes, other complications can appear like: mountain sickness (headache, nausea and anorexia), increased pulmonary ventilation, elevated heart rate, decreased plasma volume, decreased stroke volume and at very high altitudes pulmonary and cerebral oedema and sometimes coma (Bartsch and Saltin, 2008).

Altitude training and artificially simulated altitude environments are less risky than other ways of blood doping which are currently considered unethical (Lippi et al., 2006), debatable (Wilber, 2001; Wilber, 2007), illegal methods to enhance endurance sports performance at sea level for their ability to reproduce most physiological and adverse effects of rHuEPO abuse (Ashenden et al., 2001), legal (Ashenden et al., 2004) but not prohibited by WADA (The 2009 Prohibited List International standards, WADA, 2009a).

1.3.2.2. Gene Therapy (EPO Gene Doping)

According to WADA, ***"The transfer of cells or genetic elements or the use of cells, genetic elements or pharmacological agents to modulating expression of endogenous genes having the capacity to enhance athletic performance, is prohibited"*** and considered "gene doping" and therefore when being applied to EPO gene then it is indirect EPO doping (The 2009 Prohibited List International standards WADA, 2009a). With the development of gene transfer technology, EPO gene might be targeted as well as other genes to improve athletic performance. EPO gene doping and manipulation mimics the effects of exogenous EPO injections without the need for repeated doses (Unal and Unal, 2004; Diamanti-Kandarakis et al., 2005; McCrory, 2003). EPO gene is one of the

primary identified genes targeted for doping (Azzazy et al., 2005; Azzazy, 2010).

One of the possible approaches to transfer and activate EPO gene is by combining and associating EPO gene with a carrying vector by the intramuscular injection of an adenovirus containing EPO gene (Svensson et al., 1997). The intramuscular injection of adeno-associated virus vectors in animals can systemically deliver and produce supra-physiologic levels of EPO (Bohl et al., 2000; Walker et al., 2005). The excessive and uncontrolled secretion of EPO leads to elevated Hct levels that increase blood viscosity with higher risk of thrombosis, heart attack, liver failure, paralysis, and unexpected deaths (McCrory, 2003; Diamanti-Kandarakis et al., 2005). Experimental EPO gene therapy on baboons resulted in sharp raise in RBCs levels from 40% to 75% that leaded the researchers to have the baboons' blood regularly diluted to keep them alive (Zhou et al., 1998).

The only possible way to detect EPO gene doping is by invasive procedures using molecular methods to recognise and indentify EPO transgenes or gene transfer vectors that is logistically inappropriate to screen large numbers of athletes (Diamanti-Kandarakis et al., 2005). Haematological passports (Malcovati et al., 2003) and year round screening of athletes can help in detecting such abuse as athletes have alterations in haematological indices and reference ranges within and between seasons (Unal and Unal, 2004). The indirect haematological methods (Gore et al., 2003; Sharpe et al., 2006) and the direct EPO urine test (Lasne, 2001; Lasne et al., 2007) can be used as a screening tools for suspects and athletes who test positive followed by direct confirmatory molecular analysis for EPO gene doping (Diamanti-Kandarakis et al., 2005).

1.3.3. Exogenous EPO, Analogues and Mimetics

In mid 1980s, human erythropoietin gene was cloned (Lee-Huang, 1984) and by mid 1990s EPO has become the drug of choice among endurance athletes to improve their aerobic competitive performance (Cazzola, 2002). Technically, EPO doping has several advantages compared to blood transfusions like: the availability of blood donors, withdrawals, storage and reinfusion and delay in

training and decay in performance after blood withdrawal (Lippi et al., 2006). The abuse of EPO in international competitions as an ergogenic aid has been accentuated by a number of disgraces and dishonours with approximately assessed use by 3% to 7% of elite professional endurance athletes (Wilber, 2002). EPO improves muscle oxygenation by increasing the circulating RBCs via promoting, differentiating and proliferating the erythroid progenitor cells (Pascual et al., 2004). EPO abuse for doping purposes have been supported by clinical evidence for its erythropoietic and ergogenic benefits owing the significant enhancements of Hb, Hct, VO_2 max, and increases in the duration of exercise endurance time (Wilber, 2002). Different biologically active isoforms of recombinant human EPO (rHuEPO) have been developed and were named "Epoetin"s for the preparations that have an amino acid sequence similar to the naturally occurring endogenous EPO with the addition of the Greek letters α, ß, ω and δ as suffixes to distinguish among the chemical composition and/or the nature of the added carbohydrate moieties (Pascual et al., 2004). Clinically, rHuEPO have been and being used primarily to treat and correct anaemia associated with chronic kidney failure in patients undergoing renal dialysis and secondary anaemia progressed by AIDS, cancer, hepatitis C infection, chronic infections, heart failure, autoimmune diseases, transplantation of bone marrow and other seriously ill pre- and postoperative patients (Bunn, 2007; Lamon et al., 2010).

After the development of recombinant DNA techniques and the expression of human EPO gene, Epoetin α (Epogen®, Epoade®) and Epoetin ß (Epoch®, Eritrogen®) are produced and isolated from Chinese hamster ovary (CHO) cell lines with minor structural differences but similar physiological effects (Pascual et al., 2004; Diamanti-Kandarakis et al., 2005). Epoetin ß has probably slight pharmacokinetic advantages, over Epoetin α, for instance a longer terminal elimination half-life for its increased molecular weight and the decrease in the amount of sialylated glycan remnants (Storring et al., 1998; Deicher and Hörl, 2004). Epoetin ω (Epomax®and Hemax®) is a sialoglycoprotein obtained from baby hamster kidney (BHK) cells and differs from Epoetin α and ß by its physiochemical properties with similar pharmacological effects (Diamanti-Kandarakis et al., 2005). Epoetin ω being more biologically active than Epoetin α, but this was concluded by small-scale clinical studies (Deicher and Hörl,

2004). Epoetin δ (Dynepo®) or Gene-Activated EPO (GA-EPO) is produced by gene activation in human firbrosarcoma cell lines, which are engineered to induce the human endogenous EPO gene by transfecting its DNA (Diamanti-Kandarakis et al., 2005; Barbone et al., 1999). The pattern of glycosylation and the sequence of amino acids of endogenous EPO will possibly vary from the ones for Epoetin δ because it is not produced by renal cells (Diamanti-Kandarakis et al., 2005).

The need for less frequent doses of exogenous EPO directed the search towards the structural characteristics of endogenous EPO and the in vivo potency of EPO that leaded to the discovery and development of the Novel Erythropoiesis-Stimulating Protein (NESP), also known as Darbepoietin α (Aranesp®) (Diamanti-Kandarakis et al., 2005; Lamon et al., 2010; Elliott, 2008). NESP is a glycoprotein hormone containing 5 N-liked carbohydrate chains instead of 3 that makes it different from endogenous EPO and other rHuEPO's by 5 amino acids substitutions resulting in the addition of 2 N-linked oligosaccharide attachment sites and leading to weaker binding to EPO receptor and extended serum half-life (Elliott, 2008; Lamon et al., 2010; Diamanti-Kandarakis et al., 2005). Continuous erythropoietin receptor activator (CERA) is an erythropoiesis-stimulating protein that can be administered at less frequent dosage of once every three to four weeks (Macdougall, 2005). The main criteria of CERA's mechanism of action is the weaker binding to EPO receptors and even more rapid dissociation from these receptors that increases its plasma half-life (Macdougall, 2005). The painful routs of administration of rHuEPO intravenously and subcutaneously leaded research to develop a more suitable way for the simplicity, ease and convenience of use by oral EPO analogues (Diamanti-Kandarakis et al., 2005). EPO mimetics, which are substitutes of EPO, are synthetic molecules with the capability to dimerise EPO receptors and act similarly as EPO and rHuEPO (Pascual et al., 2004). Hematide ® is a synthetic dimeric and EPO mimetic peptide that activates EPO receptors, increases and prolongs EPO serum half-life without producing anti-EPO antibodies (Diamanti-Kandarakis et al., 2005; Elliott, 2008). Encapsulated rHuEPO in genetically engineered cells with semi-permeable membrane polymers prevents antigen identification and immune system rejection by isolating the encapsulated cells that enhanced erythropoiesis in healthy subjects (Regulier et al., 1998). Encapsulated rHuEPO in dipalmitoylphosphatidylcholine (DPPC) liposomes made

up of soybean-derived sterylgluciside (SG-liposomes) and soybean-derived sterols (SS-liposomes) were administered in rats and compared to subcutaneous injections and the results showed a maintenance of EPO activity by the liposomes that reduced the aggregation of EPO molecules with higher retention of SG-liposome encapsulated rHuEPO activity than SS-liposomes (Qi et al., 1995).

The pharmacokinetics, following subcutaneous rHuEPO administration, appear to be linear for dosages from 50 to 1000 $IU.kg^{-1}$ but not for a lower dosage of 10 $IU.kg^{-1}$ with mean half-life for a 50 $IU.kg^{-1}$ dose repeated daily of about 3.5 hours and total clearance, about three times higher in athletes, of 17 $ml.h^{-1}.kg^{-1}$ for athletes and 6.5 $ml.h^{-1}.kg^{-1}$ for untrained subjects (Varlet-Marie et al., 2003a; Varlet-Marie et al., 2003b). The half life is five times shorter following intravenous administration (Varlet-Marie et al., 2003b). A delayed increase of Hb (up to 9.6%) ,Hct (up to 8.3%), EPO in serum, macrocytes, Retics, and sTfr levels is noticed after rHuEPO administration (Parisotto et al., 2000a; Connes et al., 2003). Following the initial administration, these markers (Parisotto et al., 2000a) increase significantly from the third to the tenth day and at all the times continually followed by a negative temporary feedback of EPO synthesised endogenously which can be interpreted and considered as an indirect marker of rHuEPO administration (Varlet-Marie et al., 2003a) Epoetin δ has mean plasma half-life of 7-12 hours when injected intravenously and 18-20 hours of subcutaneous administration (Deicher and Hörl, 2004). Darbepoietin-α has mean half-life of approximately two to three times longer than it is for rHuEPO; 25.3 hours when administered intravenously and 48.8 for the subcutaneous administration route (Macdougall et al., 1999). Darbepoietin-α has a longer terminal elimination half-life than Epoetin α and β ranging from 6.8-8.5 up to 25.3 hours when administered intravenously and 19.4-24.2 up to 48.8 hours once administered subcutaneously (Macdougall et al., 1999). After less frequent dosages, darbepoietin-α showed to have an increased residence time with equivalent pharmacodynamic effects, of elevating Hb levels, compared to Epoetin α (Macdougall et al., 1999). The required dosage of Darbepoietin-α do not differ between the subcutaneous injection and intravenous administration with less frequent administration compared to other Epoetins (Macdougall et al., 1999). CERA has extended plasma half-life estimated and reported to be seven times longer when compared to other standard rHuEPOs with maintained Hb and

Retics values for 12 weeks at a dose of 5-8 µg (Macdougall, 2005). At this dose, CERA could increase Retics by 262% and Hb from 16-23 $g.L^{-1}$ with over 50% of anaemic patients having Hb ≥20 $g.L^{-1}$. CERA has mean elimination half-life of 133 to 137 hours for both subcutaneous and intravenous administrations (Macdougall, 2005).

Athletes misusing erythropoiesis-stimulating proteins and agents may put their cardiovascular system at great risk (Lippi et al., 2006; Elliott, 2008). With evidence based positive effects of rHuEPO therapy on physical performance, side effects and risks arose including hypertension, thrombosis, of the vascular access, and hyperviscosity, dehydration, thrombosis, hypertension and severe headache (Raine, 1988; Eagleton and Littlewood, 2003; Elliott, 2008; Sikole et al., 2002).The long term rHuEPO therapy in patients can cause pure red cell aplasia, and the development of anti-EPO antibodies (Casadevall et al., 2002; Eagleton and Littlewood, 2003; Casadevall, 2003). RBC aplasia is a condition characterised by an arrest in RBCs production which is rare but life-threatening condition (Deicher and Hörl, 2004; Eagleton and Littlewood, 2003; Casadevall, 2003; Casadevall et al., 2002).

1.3.4. O_2 Delivery Enhancement by Using Artificial O_2 Carriers (Blood Substitutes)

According to WADA, any method ***" Artificially enhancing the uptake, transport or delivery of oxygen, including but not limited to perfluorochemicals, efaproxiral (RSR13) and modified haemoglobin products (e.g. haemoglobin-based blood substitutes, microencapsulated haemoglobin products)"*** is considered a doping method (The 2009 Prohibited List International standards, WADA, 2009a). Artificial O_2 carriers or blood substitutes are synthetically made solutions and bioengineered to be capable of binding, transporting and unloading O_2 molecules from the lungs to body tissues as RBCs does (Farrar and Grocott, 2003). Currently, almost all artificial O_2 carriers are in advanced clinical trails (Kim and Greenburg, 2004; Henkel-Honke and Oleck, 2007). As an alternative to EPO Doping and blood transfusions, these solutions may possibly be used in the sports fields.

The main idea of manufacturing blood substitutes is to replace the O_2 delivery function of RBCs by overcoming and avoiding the logistical problems and disadvantages of whole blood transfusions including: storage, refrigeration, cost, immunological reactions, blood group and type matching, blood born diseases and infections, and the immediate supply of large blood quantities in severe blood loss cases of trauma, anemia and tissue ischemia (Kim and Greenburg, 2004; Henkel-Honke and Oleck, 2007; Corrigan, 2002; Lowe, 2003).

1.3.4.1. Exogenous and Synthetic Allosteric Modifiers of Hb

Some drugs where developed to modify the allosteric properties of Hb and work in synergy with 2,3-diphosphoglycerate (2,3-DPG), the native allosteric effector of Hb. Among these drugs, the analogous of clofibrate, bezofibrate and indanyl derivative known as RSR-13, RSR-4 and RSR-46, respectively being the most potent and prompt modulators to decrease the affinity of Hb to O_2 and release of it to hypoxic tissues (Kunert et al., 1996; Hou et al., 2004; Phelps Grella et al., 2000). Pharmacologically, bezofibrate and clofibrate derivatives (RSR derivatives) are the most potent synthetic allosteric Hb modifier agents (Safo et al., 2001; Phelps Grella et al., 2000; Eaton et al., 2007; Papassotiriou et al., 1998; Abraham et al., 1992) with RSR-13 being on the top lead of the clinical trials. The primary goal of manufacturing these drugs was after the need for tissue oxygenation and increasing O_2 tension in tumor tissues with hypoxic cells to increase the sensitivity and response of cancer patients to radiotherapy (Hou et al., 2004; Safo et al., 2001).

When RSR-13 binds to RBCs membrane, without damaging the cell membrane, it decreases the affinity of Hb to O_2 and thus more release of O_2 molecules to tissues by shifting the O_2-Hb dissociation curve to the right (Phelps Grella et al., 2000; Eaton et al., 2007; Papassotiriou et al., 1998). RSR-13 interacts with Hb leading to raised peripheral tissues oxygenation that can be indicated to treat tissue ischemia, hypoxia and in cases of blood loss caused by trauma, bleeding and various types of anaemia (Safo et al., 2001). RSR-13 is in phase III clinical trials with current failure to comply with drug safety and initial goal of clinical indication, use and manufacture (Safo et al., 2001; Elliott, 2008). As a possible ergogenic aid and Doping agent, RSR-13 should be used at the day of

competition. With traces up to 24 hours of last administration, RSR-13 has a short half-life of 4 to 5 hours with easy delectability in urine by gas chromatography/mass spectrometry (Breidbach and Catlin, 2001).

1.3.4.2. Perfluorocarbon Based Emulsions (PFCs)

Perfluorochemicals (PFCs) are highly fluorinated hydrocarbons with the ability to dissolve and carry blood gases, especially O_2 and CO_2, to various body tissues (Henkel-Honke and Oleck, 2007; Lowe, 2003) rather than binding to theses gases as native Hb does (Gaudard et al., 2003). PFCs have hydrophobic nature and therefore they were made into emulsions to dissolve blood gases (Kim and Greenburg, 2004). The emulsified liquid form of PFCs can be safely introduced in the body as a temporary solution for tissue ischemia (Lowe, 2003). Based on and following Henry's law, PFCs' ability to carry and deliver O_2 is in equilibrium with O_2 partial pressure (pO_2) (Spiess, 2009). Thus, PFCs can act as prompt O_2 carrier only at high blood O_2 concentration. At low pO_2, the linear relationship between PFCs and blood O_2 concentration compromises the O_2 dissolving and delivery ability of PFCs which makes native Hb, which has a sigmoidal O_2 binding relationship with O_2, a better tissue-oxygenating agent (Elliott, 2008).

Fluosol-DA was the first O_2 carrier drug to be developed as Perfluorocarbon emulsion in 1980's (Kerins, 1994). The first generation PFCs have a 3 to 4 hours half-life in the blood circulation and are cleared from the body within 7 days exhaled air (Flaim, 1994). The second generation PFCs were made in better injectable form and in smaller emulsion particles for better tissue oxygenation (Spiess, 2009; Lowe, 2003). Oxygent, a second generation PFC, has a longer half-life of up to 9 hours in the blood circulation and a total clearance from the body within 4 days (Spahn et al., 1999; Spahn, 1999).

To be used as sports doping agent, PFCs administration should be accompanied with continuous highly concentrated O_2 supplementation that is impractical and incompatible in the official sports fields and events (Lippi et al., 2006; Eichner, 2007; Elliott, 2008). Owing the fact that PFCs are not metabolized by the body and eliminated mainly by the lungs (Lippi et al., 2006; Elliott, 2008), the detection method is simply by measuring PFCs concentration of the exhaled air

using thermal vapor analyser (Shaffer et al., 1997) or by infrared absorption method (Mazzoni et al., 1999).

1.3.4.3. Haemoglobin Based O_2 Carriers (HBOCs)

Haemoglobin Based O_2 Carries (HBOCs) are cell free haemoglobin molecules which are obtained from bovine blood, human blood, or recombinant human DNA by genetic engineering (Corrigan, 2002). As blood substitutes, HBOCs were developed to replace O_2 delivery functions of RBCs and decrease the need for donated blood supplies in cases of trauma and during surgeries (Chang, 2000), ischemic vascular and life threatening advanced conditions (Weiskopf, 2010). Native Hb is a tetramer consisting of four subunits: two α-subunits and two ß-subunits (Varlet-Marie et al., 2004). Infused cell free Hb dissociates into smaller α and ß dimmers with short intravascular half-life and increased renal clearance and nephrotoxicity (Standl, 2001). In addition, cell free Hb has high O_2 affinity cause by the decresed contact with phosphates that leads to shifting the O_2-dissociation curve to the left (Chen et al., 2009). Consequently, biochemical modification of cell free Hb is essentially required to overcome the above mentioned complications to act as efficient blood substitute. These techniques started and developed with intermolecular and intramolecular cross-linking of Hb tetramers, conjugation with macromolecules, polymerisation and encapsulation of Hb tetramers in liposomes, microencapsulation in lipid vesicles and finally progressed to nanoencapsulation of Hb tetramers in biodegradable polymers (Chen et al., 2009; Gaudard et al., 2003; Standl, 2001; Chang, 2003).

PolyHeme, HemAssist and Hemopure (HBOC-201) are examples of HBOC drugs that have progressed and advanced to phase II and III clinical trials with no current approval in Europe and USA for clinical use (Weiskopf, 2010; Chen et al., 2009). In addition to renal toxicity, the main complication of former generations of HBOCs, nitric oxide scavenging by modern HBOCs is the main complication leading to the oxidation of free Hb, systemic vasoconstriction and vascular hypertension that compromise the function of HBOC drugs to deliver O_2 to deprived body tissues (Irwin et al., 2008; Chang, 2003; Chen et al., 2009; Weiskopf, 2010; Rooney et al., 1993). Despite the fact that all HBOC drugs carry O_2 from the lungs and almost all HBOC drugs unload O_2 to tissues with coherent

evidence of efficacy in different animal models, a less consistent proof of O_2 delivery efficacy has been demonstrated in clinical trials (Weiskopf, 2010). In a study on euvolumic animal subjects, HBOC derived from bovine RBCs decreased O_2 delivery to tissues during normoxia and hypoxia and failed to act as an artificial O_2 carrier (Irwin et al., 2008).

For anti-doping testing purposes, HBOCs can be easily and directly detected in blood. The presence of HBOCs in blood is very visible for the discolouration of separated plasma or serum to deep red compared to HBOCs free plasma and serum (Gaudard et al., 2003). HBOC drugs can also be detected by electrophoresis screening technique (Lasne et al., 2004) and by chromatographic profiling (Varlet-Marie et al., 2004).

1.4. Detection of Blood Doping (Transfusions and EPO)

Homologous blood transfusion is easier to detect from autologous reinfusion because exogenous RBCs' antigens differ from donor to recipient blood and these antigens can be easily expressed and discriminated (Ashenden, 2002). Via pheynotyping certain RBC antigens, the detection of homologous blood transfusions can be achieved by the distinction of antigens of the donor and recipient RBCs by flow cytometry method using blood bank antisera combined with a fluorescent-labeled additional antibody focused in opposition to human immunoglobulin (Nelson et al., 2002; Nelson et al., 2003; Lippi and Banfi, 2006). A method was proposed to detect the autologous transfusions in cross-country skiers using the combinations of blood parameters of Hb, bilirubin, iron and serum EPO of autologous reinfusion of refrigerator-stored blood but resulted in false negatives of 50% (3 athletes) of the blood-doped group (Berglund et al., 1987). The same method was applied to endurance athletes during the first four weeks of reinfused thawed freezer-stored blood (RBCs) with no robust evidence of blood doping using autologous blood transfusions (Berglund et al., 1989). To date, there is no standard and practical method to detect autologous whole blood or RBCs transfusions (Lippi and Banfi, 2006; Morkeberg et al., 2009a).

r-HuEPO abuse detection is always difficult for its short half-life and the variability in interindividual drug compounds' pharmacokinetics of the

intravenous and subcutaneous routes (Gaudard et al., 2003; Diamanti-Kandarakis et al., 2005; Lamon et al., 2010; Wilber, 2002). Several published methods were developed to detect rHuEPO abuse directly (Wide et al., 1995; Lasne, 2001; Lasne and de Ceaurriz, 2000; Lasne et al., 2007) indirectly (Wilber, 2002) and indirectly using multiparametric models (Parisotto et al., 2000a; Parisotto et al., 2001) but only two methods, which are by Parisotto et al, 2000a and in combination with lasne and de Ceaurriz, 2000 and lanse, 2001, were adopted by IOC at the Sydney Olympic Games 2000 and the Salt Lake City Winter Olympic Games 2002 (Lamon et al., 2010). To prevent bacterial activity in urine, a stability test was added by WADA for all urine samples obtained for r-HuEPO screening (Lamon et al., 2010). Epoetin α , β, ω and NESP (darbepoietin-α) are all detectable using the same methods whereas GA-EPO or Epoetin δ (Dynepo®) could probably be very difficult to detect by the direct method because of its similar structure to endogenous human EPO (Diamanti-Kandarakis et al., 2005).

1.5. Development of EPO and Blood Doping Statistical Models and Equations

Endurance competitions and events like cycling, cross-country skiing and long distance running are the games in which “blood doping” is used and preferred. Endurance athletes are usually tested for their serum and urine EPO levels. It was easier to detect the totally synthetic EPO but that was only possible in the old synthetic version where it leaves traces in blood and urine. Physiologically, EPO is released by the kidneys and the pharmaceutical EPO is made and designed for renal failure patients who undergo renal dialysis. EPO is usually lost after renal dialysis and that was the medical urge to manufacture, improve and imitate the naturally occurring human EPO. Recently, with new technology and developed pharmaceutical sciences and techniques, a new version of the hormone EPO have been released as r-HuEPO, as mentioned earlier (Parisotto et al., 2000a; Lippi et al., 2006; Gaudard et al., 2003). The tests which were developed to detect EPO abuse were unable to distinguish between the injected exogenous EPO and the endogenously produced EPO (Parisotto et al., 2000a). Therefore, some other blood parameters like red blood cells count (pack cell volume) or Hct and Hb concentration have been considered by some federations

(Parisotto et al., 2000a), like the International Cycling Union (UCI) in the late 20th century for anti-doping purposes and medical reasons after the causalities of some doped athletes who ended up dying in the competition and sports fields (Eichner, 1992; Shaskey and Green, 2000; UCI, 2009). Later on, other parameters and blood contents have been considered to help in the detection of EPO and blood doping. Recticulocytes (immature red blood cells) Haematocrit (RetHct) and sTfr concentration, in addition to Hct and Hb have been used and applied in certain models and equations according to their normal existing values and were compared to values that most athletes posses (Parisotto et al., 2001; Parisotto et al., 2000a; Parisotto et al., 2000b). Parisotto et al. 2000 and 2001 designed equations that include and relate these blood parameters and their values according to the time of EPO doping method and course, i.e. during and after the cessation of the doping procedure. These equations have been called the ON-model and OFF-model equations and are almost specific to EPO doping rather than other ways of blood doping. In Sydney 2000 Olympic Games and up to date, the ON-model in combination with the positive urine EPO sample (e.g. Lasne and de Ceaurriz, 2000) were agreed on by the IOC and later on by WADA and some federations like the International Associations of Athletics Federations (IAAF). That was the most important and first step in the detection of blood doping and the application of blood sample testing. IOC medical committee used to depend on and trust urine tests alone and that is why they did not agree on the blood test alone and excluded the OFF-model outcomes. The ON-model outcome combined with urine EPO concentration (traces) results were very weak tools, at that time when these models and urine detection methods were developed, to detect all EPO doped athletes because most r-HuEPO traces in serum and urine are cleared out from body systems between 24 to 72 hours (Bressolle et al., 1997; Souillard et al., 1996).

Following 2000 Olympic Games, there was an urgent need to modify and develop more models and equations. The first generation of ON- and OFF-models (Parisotto et al., 2000a) were insensitive and unspecific in relation to the percentage of false positives. Most elite long distance runners are born, raised, lived and trained in high altitude geographic areas but they also descend to compete on sea level fields. These athletes already have high blood parameters values and consequently higher ON- and OFF-model scores compared to other

athletes who originate from low altitudes (at or close to seal level). It was also found that an adjustments and alterations of these athletes' blood parameters and profiles when they change their altitude, as a compensatory homeostatic responses (Moore et al., 2007). Second generation ON- and OFF-models have been developed by Gore et al. to overcome these false positives and minimise them by 1 in 1,000 incidence (Gore et al., 2003). These new models have been developed in accordance to the time of season, venue, and altitude of the collected sample. WADA's policy is to track and obtain urine and blood samples of competing registered athletes in all conditions, i.e. out of completion (while training or resting in at home), pre-competition and in-competition. The third generation model was developed by Sharpe et al. that can monitor athletes hematological profiles in a longitudinal way (Sharpe et al., 2006). These third generation equations are based on the OFF-hr scores (from the second generation developed by Gore et al., 2003) and Hb concentration values of the same athlete of the same and previous seasons (Sharpe et al., 2006). Some federations like: the International Ski Federation (FIS)(FIS, 2009), UCI (UCI, 2009) and IAAF (IAAF, 2009; Ojiambo et al., 2008), in cooperation with WADA and anti-doping researchers' suggestions, started to process, develop and formulate what is so called "Athletes Haematological Passports" proposed by Malcovati et al. (Malcovati et al., 2003) and blood profiles of skiers (Morkeberg et al., 2009b), cyclists (Morkeberg et al., 2009c) and endurance runners (Ojiambo et al., 2008). These passports will include all athletes' information, all collected samples data of all conditions until the accreditation of the current OFF-models (Gore et al., 2003), and the techniques of sample collection procedures as the direct detection methods of "blood doping".

1.6. Haematological Passports

With the development of the third generation model (Sharpe et al., 2006) and haematological passports for any given endurance athletes (Malcovati et al., 2003), it is the only possible way to find out and detect blood doping using rHuEPO and autologous blood transfusions in regards to anti-doping rules stated by WADA for abnormal haematological high values of non pathological reasons. Athletes of endurance sports especially in IAAF, UCI and FIS should provide at least four blood samples a year, pre-competition, and inform their closest

National Anti-Doping Organization (NADO) of their whereabouts (Hanstad and Loland, 2009).

1.7. Masking Substances and Methods

After achieving the desired Hct and Hb concentrations, blood doped athletes usually use blood thinners, anticoagulants, anti-hypertensive drugs and plasma volume expanders. Aspirin, heparin, beta blockers, normal saline and increasing the fluid intake before, during and after the event and just before sample collection, which are the usually used procedures to prevent any medical complications and mortalities while competing and as masking methods to dilute the blood sample.

According to WADA any masking method or substance that mask a doping substance physically and or chemically by tampering, intravenous infusions and/or oral intake to dilute the urine or blood sample including but not limited to:***"Diuretics, probenecid, plasma expanders (e.g. intravenous administration of albumin, dextran, hydroxyethyl starch and mannitol) and other substances with similar biological effect(s)"*** ; violates the world anti-doping code and considered doping (The 2009 Prohibited List International standards, WADA, 2009a). Glycerol has been recently added to the 2010 prohibited substances list just after the completion of the experiments of this thesis and the publication of Easton et al., manuscript (The 2010 Prohibited List International standards, WADA, 2010; Easton et al., 2009).

1.8. Hyperhydration and Blood Dilution as a Masking Method

The reason for prohibiting the use of plasma expanders is for their direct dilution effect on plasma and therefore a diluted blood (haemodilution) sample which lower the doping substances concentrations in blood, below the threshold, that can be considered as coherent evidence to accuse, ban and apply sanctions and punishments to athletes who are doped (figure 1.2.). Hct% and Hb concentration are used to calculate plasma volume indirectly (Dill and Costill, 1974). Proper hydration by euhydration (Fudge et al., 2008) and hyperhydration (Zorbas et al.,

1995) may help endurance athletes to perform better in endurance events in contrast to hypohydration (Sawka et al., 1992a; Sawka et al., 1992b) and dehydration (Cheuvront and Haymes, 2001) especially when exercising and competing in environments with elevated temperatures.

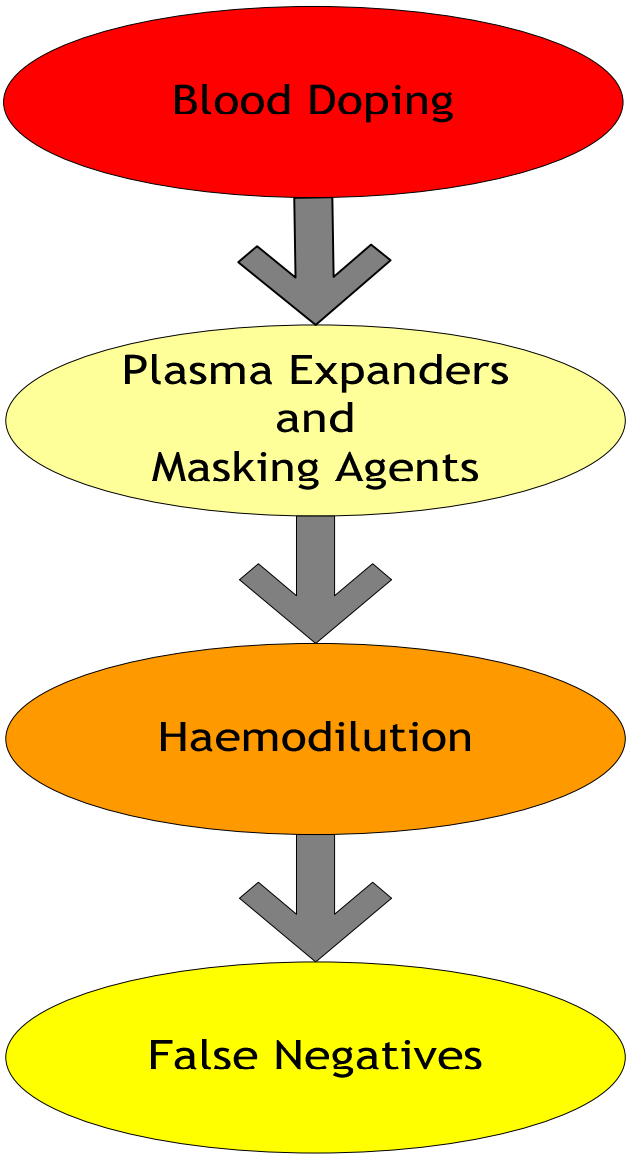

Figure 1.2 Indirect Athletes' Blood Sample Manipulation. (Masking Substances and Methods).

1.9. Glycerol(Gly) and Creatine(Cr) as masking substances

Glycerol(Gly) supplementation may be considered as an ergogenic aid by means of hyperhydration (Nelson and Robergs, 2007; Robergs and Griffin, 1998). Supplementation with Creatine (Cr) induces ergogenic effects directly on the anaerobic pathway of energy supply (Greenhaff et al., 1994; Kilduff et al., 2002; Kilduff et al., 2003). However, Cr supplementation also attenuates the effect of heat on exercise (Kilduff et al., 2004; Hadjicharalambous et al., 2008; Easton et al., 2007) by means of hyperhydration too.

The combination of Cr/Gly supplementation showed synergistic effects by means of hyperhydration that improved exercise performance in heat (Easton et al., 2007) and with different postures (Easton et al., 2009) that attenuated orthostatic intolerance too; when these studies showed significant increases in total body water (TBW) and plasma volume that were calculated using Hct and Hb (Dill and Costill, 1974). Specifically, Gly increased extracellular water (ECW) and Cr increased intra cellular water (ICW) (Easton et al., 2007; Easton et al., 2009).

1.9.1. Creatine (Cr)

Creatine (Cr) or creatine monohydrate has been used at supra-physiological level doses worldwide not only by professional elite athletes but also by recretionl sports individuals seeking improved physical performance (Bemben and Lamont, 2005) in both supra-maximal short term as well as sub-maximal prolonged exercise and competitions in different climatic situations. In the early 1830s, Chevreul discovered Cr and by 1847 Liebeg confirmed and theorised that Cr is an existing article in meat and in some way associated with muscle performance but the scientific investigation for Cr extraction and processing from meat for oral consumption did not start until the beginning of 1900s (Bemben and Lamont, 2005). By the 1970's, some eastern European countries started to use Cr as an ergogenic aid for athletic performance; and by early 1990s, scientific studies in UK and USA started to focus extensively on Cr supplementation on potential performance (Silber, 1999). Cr has been proven scientifically and legally to date in the enhancements of high to even maximal intensity exercise performance in

sports requiring single, multiple or repetitive bouts like weight lifting, spiriting, football and similar sports (Casey and Greenhaff, 2000). However, the focus in the following paragraphs and chapters will be mainly on the hyperhydration capabilities of Cr supplementation on haemodilution rather than the ergogenic effects. Brief introduction of biochemical reactions, physiological responses, effects and side effects of Cr can better help in the understanding of how Cr loading for 7 days at a dose of approximately 20 $g.day^{-1}$ can produce such hyperhydration results and maybe plasma expansion and haemodilution effects.

Cr is a nonessential naturally occurring dietary compound that can be ingested exogenously by eating meat or fish and can also be synthesised endogenously in human body mainly by the liver (Bemben and Lamont, 2005). Cr is known biochemically as methylguanidine-acetic acid and made up of 3 amino acids glycine, arginine and methionine (Casey and Greenhaff, 2000; Bemben and Lamont, 2005). At first, glycine and arginine are joined to produce guanidinoacetate and after that S-adenosylmethionine adds a methyl group for the production of Cr (Bemben and Lamont, 2005). After the synthesis of Cr mainly in the liver, it is released into the blood stream and then transported into the muscle fibers and sarcoplasm by active transport which is facilitated by a sodium-dependent transporter known as Cr transporter-1 (Demant and Rhodes, 1999; Terjung et al., 2000). The skeletal muscle tissue is considered the main host that contains almost all the Cr ingested and synthesised in human body in which approximately 60% is phosphorylated and known as phosphocreatine (PCr) with the remaining 40% in free form (Bemben and Lamont, 2005). An average human body of 70 kg person generally posses 120-140 g of Cr in the skeletal muscle tissues and about 2 $g.day^{-1}$ of this pool is lost and excreted in the urine as creatinine that is restored by the endogenous synthesis of about 1 $g.day^{-1}$ and further exogenous 1 $g.day^{-1}$ from oral ingestion of normal balanced diet of mixed food (Bemben and Lamont, 2005). As a dietary supplement, Cr ingestion as an ergogenic aid can operate through different but related mechanisms on the skeletal muscle tissue (Bemben and Lamont, 2005). Once intracellular PCr levels are raised by the uptake of Cr into muscle fibers, it facilitate the rapid rephosphorylation of ADP molecules back to ATP via the action of Cr kinase during short duration high intensity exercise and particularly if that exercise is repeated and separated by short rest intervals (Casey et al., 1996a; Demant and

Rhodes, 1999; Mesa et al., 2002). Cr availability into sarcoplasm and myofibrils facilitates the high-energy phosphate molecules movement between myosin heads (thick filaments) and mitochondria and enhances the sarcoplasmic reticulum to uptake Calcium ions that lead to better occupying the heads of the thick filaments in crossbridge binding and cycling and in sustaining the tension during muscular contraction (Bemben and Lamont, 2005; Demant and Rhodes, 1999; Mesa et al., 2002; Casey et al., 1996b; Leemputte et al., 1999; Hespel et al., 2002; Wyss and Kaddurah-Daouk, 2000). As PCr levels decline with exercise and ADP levels start to rise, it can stimulate the glycolysis rate-limiting enzyme, known as phosphfructokinase, to rapidly raise the process of rephosphorylation of ADP to ATP (Bemben and Lamont, 2005; Demant and Rhodes, 1999; Mesa et al., 2002). However, this rephosphorylation process is further enhanced and accelerated by the presence of free and phosphorylated Cr within the muscular tissue in improving the intracellular homeostasis via stimulating Cr kinase reactions by acting as a pH buffer in raising the acidosis and making the most of using hydrogen ions (Mesa et al., 2002; Demant and Rhodes, 1999; Bemben and Lamont, 2005). Cr supplementation has been found to decrease the concentrations of the accumulated plasma ammonia (Birch et al., 1994) and hypoxanthine (Balsom et al., 1993) during muscular metabolism in sustaining the rephosphorylation process of ADP to ATP during high intensity exercise despite the amplified work and torque produced by the skeletal muscle contractions (Casey et al., 1996a; Casey and Greenhaff, 2000). Skeletal muscle hypertrophy and elevated protein synthesis rate can be linked to elevated PCn levels that are induced by Cr ingestion and high intensity exercise (Bemben and Lamont, 2005; Casey et al., 1996a; Casey et al., 1996b; Casey and Greenhaff, 2000). Universally, it appears that there is an agreement in the literature that free Cr and PCr are higher in fast twitch type II glycolytic skeletal muscle fibers compared to slow twitch type I aerobic skeletal muscle fibers with modest scientific evidence that support the differences in PCr levels between males and females (Mesa et al., 2002; Pastoris et al., 2000; Casey et al., 1996a; Casey et al., 1996b).

As a result of the loading effect of Cr (from 5-7 days), protein synthesis in the skeletal muscle tissue can be stimulated and protein catabolism can be inhibited by the osmotic gradient that moves the water from the extracellular

compartment into the intracellular compartment of the skeletal muscle cells (Haussinger et al., 1993). This increased ICW has an effect on increasing body mass in most subjects whereas some authors consider it controversial (Poortmans and Francaux, 2000; Kilduff et al., 2002; Kilduff et al., 2003).

The use of Cr supplementation by athletes maybe not safe on the long run and certain precautions should be followed to avoid the few adverse effects that have been cited as to the precise timing and the preparation of fluid supplements containing Cr (Bemben and Lamont, 2005). To prevent the degradation of Cr to creatinine, Cr should be dissolved in warm water or fluid mixture and the solution should be made fresh prior to oral consumption (Easton et al., 2007; Kilduff et al., 2003). Cr should be well dissolved in the supplementation fluid before ingestion to prevent gastrointestinal distress and stomach upset (Mesa et al., 2002). Gastrointestinal distress can occur from Cr consumption during or immediately following exercise (Vanderberie et al., 1998). However, no gastrointestinal distress was reported in a study by Kilduff et al. when Cr was supplemented immediately after exercise (Kilduff et al., 2003). Cr supplementation does not affect or impair the liver or kidneys physiological function but caution should considered in individuals and athletes with any pre-existing conditions and associated pathologies of the renal or hepatic functions especially with long-term use (Bemben and Lamont, 2005). Muscle cramps are sometimes experienced with Cr supplementation after exercise but this is possibly related to the high intensity of the exercise (Terjung et al., 2000; Mesa et al., 2002) or may be due to intramuscular electrolyte imbalance and dehydration (Bemben and Lamont, 2005). No muscle cramps have been reported with Cr supplementation with high intensity workout when the subjects were adequately hydrated and hyperhydrated (Kilduff et al., 2003).

1.9.2. Glycerol (Gly)

Glycerol(Gly) has been recently considered a banned substance (The 2010 Prohibited List International standards, WADA, 2010). Gly (1,2,3- propanetriol) is the metabolite of a 3-carbon alcohol which is naturally synthesised in the human body, found in and between all body cells at low concentrations of less than 0.1 $mmol.L^{-1}$ (Robergs and Griffin, 1998) and known to be the main structure that

makes up a triglyceride molecule (Frank et al., 1981). Gly can be formed from the action of glycerol kinase on glycerol-3-phosphate (Robergs and Griffin, 1998) and by the de-esterification of a triglyceride molecule found in specific blood vessels, the adipose tissue, the liver and the skeletal muscle tissue (Frank et al., 1981). The synthesis, catabolism and metabolism of Gly are mediated particularly by the enzymes glycerol kinase and glycerol dehydrogenase which are tissue target specific and mostly found in the kidneys and the liver (Bortz et al., 1972) with smaller concentrations found in the intestinal mucosa (Frank et al., 1981) and skeletal muscle tissues (Newsholme and Taylor, 1969). The conversion and turnover of Gly to a glycolytic substrate occurs for consequent metabolism which are principally controlled by the renal and hepatic function and its removal from the blood circulation depends on the activity and abundance of the glycerol kinase and glycerol dehydrogenase enzymes in those specific body tissues (Robergs and Griffin, 1998). The metabolism of Gly occurs mainly (about 80%) in the liver with the remaining 10-20% metabolised in the kidneys depending on the action of the enzyme glycerol kinase and its availability (Frank et al., 1981; Nelson and Robergs, 2007). After the conversion of Gly into glycerol-3-phosphate, two pathways are possible for the metabolism of Gly (Frank et al., 1981; Nelson and Robergs, 2007). The oxidation of glycerol-3-phosphate, accounts for about 70-90% of Gly metabolic process (Frank et al., 1981; Nelson and Robergs, 2007), produces dihydroxyacetone phosphate by the action of glycerol-3-phosphate dehydrogenase enzyme that leads to either the production of glucose and 2 pyruvate molecules via gluconeogenesis (Frank et al., 1981; Berg et al., 2007) or pyruvate and 2 ATP molecules during glycolysis (Stryer, 1995; Berg et al., 2007). The second pathway of glycerol-3-phosphate metabolism, accounts for about 10-30 % of Gly metabolic process (Frank et al., 1981; Nelson and Robergs, 2007). This process occurs when two free fatty acid molecules are added to glycerol-3-phosphate to produce phosphatidate which transforms into triglyceride when an extra (third) free fatty acid molecule is added (Robergs and Roberts, 1997; Berg et al., 2007; Robergs and Griffin, 1998). The concentration of serum Gly at rest is approximately 0.05 $mmol.L^{-1}$ and can be elevated to about 0.3 $mmol.L^{-1}$ by the effect of increased lipolysis related to caloric restriction or drawn out exercise (Robergs and Griffin, 1998). Acting as a substrate, Gly is used and thought to be an ergogenic aid especially in sports requiring endurance performance. In addition, the slow rate by which

Gly is metabolically cleared from the blood circulation plays an essential role in the persistent hydrating qualities of this compound and in the process of hyperhydration as well (Nelson and Robergs, 2007).

The use of Gly by the oral and intravenous routes have been researched for about 60 years and clinically between the years 1961 and 1980 to treat cerebral oedema, intracranial hypertension, intraocular hypertension (known as glaucoma), postural syncope and to enhance rehydration especially in gastrointestinal disorders (Robergs and Griffin, 1998). Gly hyperhydration with the addition of fluids intake have been used since 1987 for the aim of increasing and elevating total body water (TBW) by about 700 ml for the purpose of improving thermoregulation and performance of endurance exercise especially in hot environments (Robergs and Griffin, 1998). The use of Gly with exercise as an ergogenic supplement for better thermoregulatory responses through hyperhydration and fluid retention have been tested and showed decreased thirst sensation and dehydration (Wingo et al., 2004) and decreased rectal temperature (Easton et al., 2007; Lyons et al., 1990), heart rate, and rate of perceived exertion (RPE) (Easton et al., 2007). In contrast, some other authors showed no significant improvements in athletic performance (Magal et al., 2003; Marino et al., 2003).

The infusion and ingestion of Gly at a dose of more than 1.0 $g.kg^{-1}$ body mass leads to increases in serum Gly concentration to about 20 $mmol.L^{-1}$ that elevate serum osmolality to more than 10 $mOsmol.kg^{-1}$ (Robergs and Griffin, 1998). Gly induces osmotic gradient between cells and tissues (Berne and Levy, 1998) because of its even distribution and availability in all body fluid compartments except for the aqueous humour and cerebrospinal fluid (Lin, 1977) that causes intracranial and intraocular dehydration especially when infused intravenously with the possibility of body hyperhydration when ingested orally at doses starting with 1.0 $g.kg^{-1}$ body mass (Nelson and Robergs, 2007). Because of the characteristic properties of the blood brain barrier, Gly has this discriminative permeability (Nelson and Robergs, 2007). When taken orally, passive diffusion is the mechanism through which Gly gets absorbed by the gastrointestinal tract (Lin, 1977). Gly acts as a solute for its hyperosmotic chemical properties and when it is absorbed it leads to raises in the concentration of the solute in all

fluid compartments rather than outside them that causes an osmotic gradient directing the fluids to move from the area of lower osmotic concentration to the area that posses higher osmotic concentration (i.e Gly) to create osmotic equilibrium outside and with in all fluid compartments; leading to the even body fluids distribution (water) in the ECW and ICW water compartments (Nelson and Robergs, 2007; Berne and Levy, 1998). However, when this distribution is not created and higher concentrations of Gly is found in the ECW compartment, the fluids are forced to move to the ECW compartment following that osmotic gradient and causing intracellular dehydration and increases in the blood volume possibly by about 2.5% for an average 70 kg human with blood volume of 5 L; that if Gly hyperhydration raised TBW of about 1 L (1000 ml) casing a raise in blood volume of about 125 ml (Guyton, 1991; Nelson and Robergs, 2007). Gly ingestion were shown to be more effective on increasing ECW rather than ICW (Easton et al., 2007).

1.10. Posture and time as masking methods

Plasma volume and some blood parameters change with postural changes (Maughan et al., 1994) especially with exercise (Greenleaf et al., 1979c; Greenleaf et al., 1979b; Greenleaf et al., 1979a; Gonzalez-Alonso et al., 1999). During the process of the development of EPO and blood doping indirect models, the supine resting postural position was chosen and standardised with time ranging between 5 to 10 min to minimise the confounding effects of posture on blood parameters and plasma (Parisotto et al., 2000a; Parisotto et al., 2001; Ashenden et al., 2001; Sharpe et al., 2002; Ashenden et al., 2003). The time course, training and altitude has their effects on plasma, blood parameters and on EPO detection models (Sharpe et al., 2002). However, WADA had standardised the blood collection posture and this is the posture followed by WADA's authorised blood collection personnel that in the Guidelines for Blood Collection; article 5.7.5 ***"In order to ensure the same conditions for all, the Athlete shall remain seated and relaxed for at least 10 minutes before undergoing Venipuncture"*** (WADA Standards and Harmonization Guidelines for Blood Collection, WADA, 2008). The time of blood and urine sample collection following exercise is very crucial and may change the validation of the sample. Recently, false positive result of NESP (darbepoietin-α) was detected in urine

sample collected following exercise whereas negative result of NESP (darbepoietin-α) in urine of a sample obtained from the same subject an hour after exercise session (Beullens et al., 2006).

1.11. Hypothesis and Aim of the Experiments

Athletes that use a number of illicit substances and methods such as blood transfusions, erythropoiesis stimulating agents and blood substitutes could resort to using masking substances and methods to overcome detection when tested by WADA personnel. Hyperhydration has the potential, therefore, to act as a masking agent. Postural changes can also have a direct effect on plasma volume and therefore could influence the outcome of doping tests. There is therefore an urgent need to examine the effects of novel hyperhydration techniques alone and in combination with postural changes on blood profiles and therefore on the potential to influence the result of doping tests. A series of experiments were designed and presented in this thesis to assess the effects of a novel hyperhydration strategy involving Cr and Gly supplementation (aimed at elevating TBW via effects on ICW and ECW) on blood parameters (e.g. Hct and Hb values). The effects of posture on these blood parameters were also assessed.

The aims of the experiments described in this thesis were to examine:

i) the effects of hyperhydration using Cr/Gly supplementation on blood parameters (Hb and Hct) in the supine posture.

ii) the effects of postural changes in euhydrated subjects moving from supine, sitting to sanding posture.

iii) the effects of hyperhydration using Cr/Gly supplementation (Cr for 7 days and Gly on the 7^{th} day only) on blood parameters (Hb, Hct, %Retics, and OFF-hr model) when adopting a standard sitting posture (WADA's standard position).

CHAPTER 2

METHODS

2. Methods

2.1. Creatine and Glycerol Hyperhydration Experiment Methods (EXP1)

2.1.1. Subjects

Eight healthy male subjects (six Europeans and two Middle Easterns), were invited to participate and volunteer in this study. Prior to the first familiarisation trial, subjects signed and gave their written informed consent to take part in the present study which was approved by the Local Ethics Committee and was performed according to the code of ethics of the World Medical Association (Declaration of Helsinki). The subjects had the following characteristics (mean ± s.d.): age: 26 ± 6 years, height: 176 ± 7 cm, weight: 73.3 ± 7.6 kg. Subjects were interviewed to confirm that all subjects were Cr free for at least 8 weeks prior to the study. The investigators did not reveal prior to the interview that subjects would be excluded if they had supplemented with Cr in the previous 8 weeks. The subjects were fully informed of any risks and discomforts associated with the experiments before giving their written informed consent to participate. Discomforts may include but not limited to painful cannulation, blood withdrawal or magnitude of commitment required. All subjects were advised to revise their medical history with their local general practitioners.

2.1.2. Experimental design

Each subject was required to visit the laboratory on at least 3 occasions for measurement and analysis of urine, blood and blood content. The supine 0^{o} postural position was chosen to comply with previous studies (Parisotto et al., 2001; Sharpe et al., 2002; Ashenden et al., 2003). However, in this study, variability in Hb and Hct were the main focus points in addition to urine osmolality. The first (familiarisation) and second (pre-supplementation) experimental trials were identical and conducted prior to any supplementation intervention to establish baseline values. The final experimental trial (post-

supplementation) was conducted after supplementation with Cr and Gly for 7 days. The supplementation regimen only commenced if physiological responses between baseline experimental trials differed by less than 5%; only one subject had to repeat a third baseline experimental trial. Cr/Gly supplementation consisted of 11.4 g of Cr·H_2O (Creatine 6000-ES, Iovate Health Sciences Research Inc., Canada) (equivalent to 10 g Cr) and Gly of 1.0 g.kg $^{-1}$ body mass (Aldrich-Chemical, Milwaukee, WO, U.S.A), flavoured with 200 ml of commercially available, sugar-free, flavoured diluting juice made up in 1 L of warm water (approximately 50^{o}C). The solution was cooled to room temperature before being consumed twice daily (once in the afternoon and once in the evening) for 6 days and once more 5 hours before the experimental exercise trial.

Each supplement was made fresh prior to consumption in order to prevent any degradation of Cr to creatinine. This fluid-loading strategy has been shown to be the most effective in a comparison of several Cr/Gly supplementation regimens (Easton et al., 2007; Easton et al., 2006). Subjects were identified and classified as "responders" to Cr supplementation if the results showed an increase in body mass by 0.2 Kg (Easton et al., 2007; Kilduff et al., 2003). Subjects were asked to maintain their normal diet and minimise caffeine intake to one cup of tea or coffee per day to lessen any possible effects of caffeine on normal diuresis (Neuhäuser-Berthold et al., 1997; Riesenhuber et al., 2006) and possible confounding effects of caffeine on Cr (Vandenberghe et al., 1996). Five hours prior to the familiarisation and pre-supplementation trials subjects were required to ingest 1 L of water to match fluid ingestion volume with the post-supplementation trial. On all of the experimental test days, subjects ingested further 500 ml of water 1 hour prior to the experimental trial in an attempt to ensure that all subjects were euhydrated (Convertino et al., 1996).

2.1.3. Procedures

Subjects arrived at the laboratory on the day of each experimental trial following a 3 hour fast and having refrained from alcohol, caffeine and strenuous exercise the day before. Upon arrival at the laboratory, subjects were asked to provide a 20 ml sample of urine in order to assess hydration status (Shirreffs and Maughan, 1998). Body mass (Avery Berkel 33/448, W & T Ltd., U.K.) and height (Leicester stadiometer, Invicta Plastics Ltd, U.K.) were measured before

subjects were instructed to lay in supine (0^{o}) postural position on a plinth for 30 min with both arms abducted to the level of the heart. During this period, subjects left or right arms were prepared to be cannulated by trained phlebotomist. Tourniquet was strapped on the right or left arm and particularly over the brachial artery (Parisotto et al., 2001; Sharpe et al., 2002; Ashenden et al., 2003). A 20 G indwelling catheter was inserted into the right or left anticubital vein by butterfly needle cannula (Biovalve, Laboratories Pharmaceutics VYGON, France) and connected to multidirectional stopcock for infusion therapy and monitoring (Discofix-2, B/BRAUN, B.Braun Melsungen AG, Melsungen, Germany), stabilised with an adhesive non-abrasive tape and flushed with 2 ml isotonic saline 0.9% Na/Cl (0.9%w/v Sodium Chloride Intravenous Infusion BP, Mini-Plasco NaCl, B/BRAUN, B.Braun Melsungen AG, Melsungen, Germany). A 10 ml blood sample was collected every 10 min. The venous cannula was kept patent by a 10 ml infusion of the isotonic saline between samples. Body water compartments were estimated using a Bodystat Multiscan 5000 Bioimpedance analyser (Bodystat Ltd, Isle of Man, U.K.) after 15 min of the supine position rest. This method allows TBW and ECW to be estimated; from these measurements ICW can also be deduced. There is good evidence to suggest that the estimation of TBW by bioimpedance is reliable and valid when subjects are euhydrated (O'Brien et al., 2002). To date, several studies have successfully utilised this technique in order to estimate hyperhydration induced changes in TBW (Easton et al., 2007; Kilduff et al., 2004). Subjects were advised to remain as stable as possible for the whole experiment time and especially before obtaining blood samples and measurement of body water compartments. All experimental trials were conducted strictly at sea level barometric pressure with an ambient temperature maintained at $23 \pm 0.5^{o}C$.

Table 2.1. Experimental Design (EXP1)

Test	Week	Supplementation
Familiarisation	Week 1	No supplements
Pre-Supplementation	Week 2	No supplements
Pre-Supplementation **(if difference more than 5%)**	Week 3	No supplements
Post-Supplementation	Week 3 or 4	**Cr + Gly for 7 days**

Table 2.2. Experimental procedures (EXP1)

Time **Posture**	**-15 min**	**0 min**	**5 min**	**10 min**	**15 min**	**20 min**	**25 min**	**30 min**
Supine (0˚)	Urine Sample and Cannulation	No Blood	No Blood	First 10 ml Blood Sample	Body water (bioimpedence)	Second 10 ml Blood Sample	No Blood	Third 10 ml Blood Sample

2.2. Postural Changes Experiment Methods (EXP2)

2.2.1. Subjects

Ten healthy subjects (two Europeans, four Middle Eastrens, one Asian, one African and two South Americans), were invited to participate and volunteer in this study. Prior to this one trail test study, subjects signed and gave their written informed consent to take part in the present study which was approved by the Local Ethics Committee and was performed according to the code of ethics of the World Medical Association (Declaration of Helsinki). The subjects had the following characteristics (mean ± s.d.): age: 27 ± 3 years, height: 170 ± 6 cm, weight: 76.3 ± 8.7 kg. Subjects were interviewed to confirm that all subjects were free from consuming substances and supplements that may retain water or having any diuretic effects. The subjects were fully informed of any risks and discomforts associated with the experiment before giving their written informed consent to participate. Discomforts may include but not limited to painful cannulation, blood withdrawal or length of the experiment time especially in standing position. All subjects were advised to revise their medical history with their local general practitioners.

2.2.3. Experimental design

Each subject was required to visit the laboratory on only one occasion for measurement and analysis of blood content. The supine (0^{o}) postural position was chosen to conform to previous studies, i.e. from 5 min to maximum and not exceeding 10 min (Parisotto et al., 2001; Sharpe et al., 2002; Ashenden et al., 2003). The sitting postural position was adopted from the WADA's only authorised posture to obtain blood samples for anti-doping purposes (WADA Standards and Harmonization Guidelines for Blood Collection WADA, 2008). However, the standing postural position was used to compare and refer both supine and sitting postures. In this study, Hb and Hct fluctuations were the only focus points. Subjects were asked to maintain their normal diet and minimise caffeine intake to one cup of tea or coffee in the day of experiment to lessen any possible effects of caffeine on normal diuresis (Neuhäuser-Berthold et al., 1997; Riesenhuber et al., 2006). On the experimental test day, subjects

ingested 500 ml of water 1 hour prior to the experimental trial in an attempt to ensure subjects were euhydrated (Convertino et al., 1996).

2.2.4. Experimental Procedures

Subjects arrived at the laboratory on the day of the experimental trial following a 3 hour fast and having refrained from alcohol and strenuous exercise the day before. Upon arrival at the laboratory, subjects were prepared to be cannulated by a trained phlebotomist. The cannulation process was performed exactly as in EXP1. Then, subjects were instructed to lay in a plinth in a supine (0^{o}) postural position. In this experimental phase subjects remained in the supine position for a further 30 min before being instructed to move into sitting postural position for 30 min and finally to standing postural position for 30 min as well. A 10 ml blood sample was collected every 10 min. The venous cannula was kept patent by a 10 ml infusion of isotonic saline between samples. Subjects were instructed to relax their muscles, especially their calf muscles, at all the postural phases. Tests were to be immediately terminated if subjects complained of dizziness or light-headedness at any time of the postural positions. In the standing postural position, subjects were standing against a wall and were provided with supporting pillows against their lumbar spines to insure that they are as comfortable as possible. All experimental trials were conducted strictly at sea level barometric pressure and with an ambient temperature maintained at 23 ± 0.5^{o}C.

Table 2.3. Experimental Design and Procedures (EXP2)

Time / Posture	-15-0 min	5 min	10 min	15 min	20 min	25 min	30 min
Supine (0°)	Cannulation	None	**10 ml blood**	None	**10 ml blood.**	None	**10 ml blood**
Sitting	None	None	**10 ml blood**	None	**10 ml blood.**	None	**10 ml blood**
Standing	None	None	**10 ml blood**	None	**10 ml blood.**	None	**10 ml blood**

2.3. Running with Creatine and Glycerol Hyperhydration Experiment Methods (EXP3)

2.3.1. Subjects

Seven European healthy male athlete subjects, who trained at least 3 times a week and who at least competed once in national competitions, were invited to participate and volunteer in this study. Prior to the first familiarisation trail, subjects signed and gave their written informed consent to take part in the present study which was approved by the Local Ethics Committee and was performed according to the code of ethics of the World Medical Association (Declaration of Helsinki). The subjects had the following characteristics (mean ± s.d.): age: 22 ± 2.5 years, height: 176 ± 6 cm, weight: 71.4 ± 8.85 kg, BMI: 23 ± 1.7 $kg \cdot (m^2)^{-1}$ and VO_2 max 58.4 ± 6 $ml.kg^{-1}.min^{-1}$. Subjects were interviewed to confirm that all subjects were Cr free for at least 8 weeks prior to the study. The investigators did not reveal prior to the interview that subjects would be excluded if they had supplemented with Cr in the previous 8 weeks. The subjects were fully informed of any risks and discomforts associated with the experiments before giving their written informed consent to participate. Discomforts may include but not limited to painful cannulation, blood withdrawal or length of the experiment time. All subjects were advised to revise their medical history with their local general practitioner.

2.3.2. Experimental design

Each subject was required to visit the laboratory on at least 4 occasions for measurement and analysis of VO_2 max, urine, blood and blood content. The sitting postural position was chosen to standardise WADA's only authorised posture to obtain blood samples from the selected athletes prior to testing (WADA Standards and Harmonization Guidelines for Blood Collection WADA, 2008). However, in this study, which also focuses on hyperhydration that might be a masking method in blood doping detection/ deterring purposes, Hb, Hct, reticulocytes (%Retics) and OFF-hr score fluctuations are the main focus points. The first trial was a VO_2 max test. The second (familiarisation) and third (pre-

supplementation) experimental trials were identical and conducted prior to any supplementation intervention to establish baseline values. The final experimental trial (post-supplementation) was conducted after supplementation with Cr for 7 days and Gly for one day only (on the 7th day). The supplementation regimen and concentrations of Cr and Gly provided were the same as in EXP1. The solution was cooled to room temperature before being consumed twice daily (once in the afternoon and once in the evening) for 6 days and once more 5 hours before the experimental exercise trial; the latter was by the addition of Gly to the mixture. Each supplement was made fresh prior to consumption in order to prevent any degradation of Cr to creatinine. Subjects were identified and classified as "responders" to Cr supplementation if the results showed an increase in body mass by 0.2 Kg (Easton et al., 2007; Kilduff et al., 2003). Subjects were asked to maintain their normal diet and minimise caffeine intake to one cup of tea or coffee per day to lessen any possible effects of caffeine on normal diuresis (Neuhäuser-Berthold et al., 1997; Riesenhuber et al., 2006) and possible confounding effects of caffeine on Cr (Vandenberghe et al., 1996). Five hours prior to the familiarisation and pre-supplementation trials subjects were required to ingest 1 L of water to match fluid ingestion volume with the post-supplementation trial. On each of the experimental test days, subjects ingested 500 ml of water 1 hour prior to the experimental trial in an attempt to ensure subjects were euhydrated (Convertino et al., 1996).

2.3.3. Procedures

Subjects arrived at the laboratory on the day of each experimental trial following a 3 hour fast and having refrained from alcohol, caffeine and strenuous exercise the day before. Upon arrival at the laboratory, subjects were asked change into preferred comfortable running clothing and to provide a 20 ml sample of urine in order to assess their hydration status (Shirreffs and Maughan, 1998). Nude Body mass (Avery Berkel 33/448, W & T Ltd., U.K.) and height (Leicester stadiometer, Invicta Plastics Ltd, U.K.) were measured before subjects were instructed to be in sitting postural position in an armed chair comfortably for 10 min. During this period, subjects' arms were prepared to be cannulated by trained phlebotomist. The cannulation process was performed exactly as in EXP1. No blood or urine samples were collected at the first test. All

10 ml blood samples were collected at room temperature (20-21°C) with the relative humidity of 30-40% after sitting for 10 min exactly. The venous cannula was kept patent by a 10 ml infusion of isotonic saline after the first, second and last samples. Subjects were advised to remain as stable as possible for the whole blood collection time i.e. 10 min. The first 10 ml blood sample was collected just before kindly moving subjects to the environmental (climate) chamber to start the first 30 min running session at 10°C (10.45 ± 0.23°C) with relative humidity of 70% (72.0 ± 1.1%) on a motorised treadmill (Woodway, Germany) at 1% grade and on a speed set to 60% of their VO_2 max. Then, the treadmill was stopped gradually and subjects were asked to step off the treadmill and were kindly removed out of the chamber. Subjects then asked to rest for 30 min. During this interval, subjects were asked to return back to sitting posture at room temperature for 10 min, without ingesting any sort of fluids, to obtain the second 10 ml blood sample. At the last 20 min of the resting period, nude body mass was obtained in order to assess the fluid loss. Subjects were asked to replace the fluid loss by the given equivalent amount of water. After this resting period, subjects were asked to move back to the environmental (climate) chamber to start the second 30 min running session at 35°C (34.72 ± 0.19°C) and with the relative humidity of 70% (71.5 ± 0.9%) on the treadmill at 1% grade and on a speed set to 60% of their VO_2 max. Then, the treadmill was stopped gradually and subjects were kindly removed from the chamber to be seated for 10 min at room temperature to obtain the last 10 ml blood sample without ingesting any sort of fluids. After obtaining the last blood sample, subjects were allowed to drink their preferred soft drinks, cool down on treadmill and offered water and showering facilities. All experimental trials were conducted strictly at sea level barometric pressure.

Table 2.4. Running with Creatine and Glycerol hyperhydration experimental design (EXP3)

Test	Week	Supplementation
VO_2 max	Week 1	No supplements
Familiarisation	Week 2	No supplements
Pre-Supplementation	Week 3	No supplements
Post-Supplementation	Week 4	**Cr** for 7 days + **Gly** on 7th day

Table 2.5. Experimental Procedures (EXP3)

Time (min)	-15	-10	0 → 30	40	50→ 70	70→ 100	120	120 →∞
Test / procedure	Urine Sample and Cannulation	First 10 ml Blood Sample	Running 30 min at 60% VO_2 max (10ºC)	Second 10 ml Blood Sample	Rest 20 min + Replace fluid loss by water	Running 30 min at 60% VO_2 max (35ºC)	Third 10 ml Blood Sample	Recovery and cooling down

2.4. Urine Analysis

Urine samples, collected just before the test started, were analysed for osmolality by freezing point depression (Micro-osmometer 3300, Vitech Scientific, West Sussex, U.K.). Urine analysis was carried out in duplicates.

2.5. Blood Analysis

Blood was drawn into dry syringes and 10 ml dispensed into a tube containing K3EDTA. The blood from these tubes was analysed for Hb by the cyanmethaemoglobin method (Sigma, Chemical Company Ltd., Dorset, U.K.) and packed cell volume (PCV) by the conventional microhaematocrit (Hct) method using microhaematocrit centrifuge (Hettich Mikro 22R- Microcentrifuge, Andreas Hettich GMBH & Co. KG, Tuttingen, Germany) and HAWAKSLEY capillary tubes and HAWAKSLEY reader for the microhaematocrit centrifuge (Hawaksley and sons Ltd, Sussex, UK). Haemoglobin (Hb) analysis was carried out in duplicate and Hct or PCV was carried out in triplicate.

2.6. Reticulocytes

Reticulocytes parameters were analysed using a haematology analyser (ABX Pentra 120, France) and measured as an absolute concentration value that is converted then presented as % reticulocytes (%Retics).

2.7. OFF-hr Score

OFF-hr score was calculated using the following second generation OFF-hr score equation:

$$\textbf{OFF-hr} = \textbf{Hb} - 60\sqrt{\textbf{(Ret)}} \qquad \textbf{(Gore et al; 2003)}$$

Whereas **Hb** represents haemoglobin concentration [$g.L^{-1}$] and **Ret** represents [%] Reticulocytes (Gore et al., 2003).

2.8. Data Analysis

Data were expressed as the mean ± s.d. following a test for the normality of distribution. Statistical analysis was carried out using two factors mixed model ANOVA with repeated measures, followed by a simple main effects analysis for two-way interactions. In addition, the magnitude of change (Δ) between experimental trials was examined using a paired t-test when significance was identified using the simple main effects analysis. All statistical procedures were completed using SPSS for Windows version 15.0 (SPSS, Inc., Chicago, IL, USA). Statistical significance was declared at ($P<0.05$).

CHAPTER 3

RESULTS

3. Results

3.1. Creatine and Glycerol Hyperhydration Experiment (EXP1)

3.1.1. Creatine Responders

All Subjects were responders to Cr supplementation with increased body mass ≥0.2 kg (Kilduff et al., 2003; Easton et al., 2007).

3.1.2. Body mass and water compartments

Body mass did not change between familiarisation and pre-supplementation tests (P=0.53) (figure 3.1.). There was a significant increase in body mass after Cr and Gly supplementation by 0.82 ± 0.22 kg compared to pre-supplementation test (P<0.05). TBW increased significantly after supplementation by 0.67 ± 0.17 L (P<0.05) with no changes recorded between familiarisation and pre-supplementation tests (P=1.000) (figure 3.1.). ECW increased significantly after supplementation by 0.35 ± 0.09 L (P<0.05) with no changes between familiarisation and pre-supplementation (P=1.000) (figure 3.1.). There was a significant increase in ICW following hyperhydration by 0.37 ± 0.15 L (P<0.05) whereas no changes were noticed between familiarisation and pre-supplementation tests (P=0.285) (figure 3.1.).

3.1.3. Urine Osmolality

Urine osmolality was significantly higher following Cr and Gly supplementation compared to the pre-supplementation tests (471 ± 252 mOsmol.kg^{-1} vs. 632 ± 137 mOsmol.kg^{-1} respectively, P<0.05) (figure 3.2.) There was no difference in urine osmolality between familiarisation and pre-supplementation test (P=0.877).

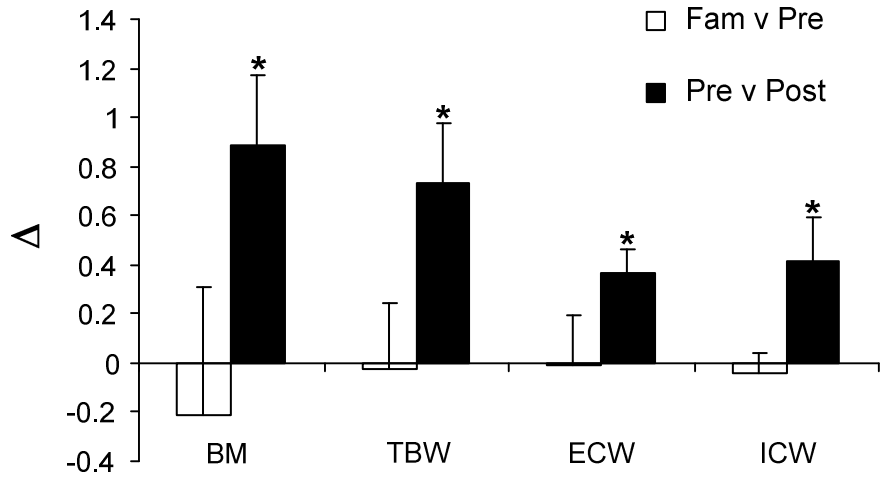

Figure 3.1. Change in Body Mass and Water Compartments (n=8). Comparisons between Familiarisation and Cr/Gly Pre-supplementation Tests (□ Fam v Pre). Comparisons between Cr/Gly Pre-supplementation and Cr/Gly Post-supplementation Tests (■ Pre v Post). Body Mass (BM). Total Body Water (TBW). Extracellular water (ECW). Intracellular Water (ICW). Significant Change (*).

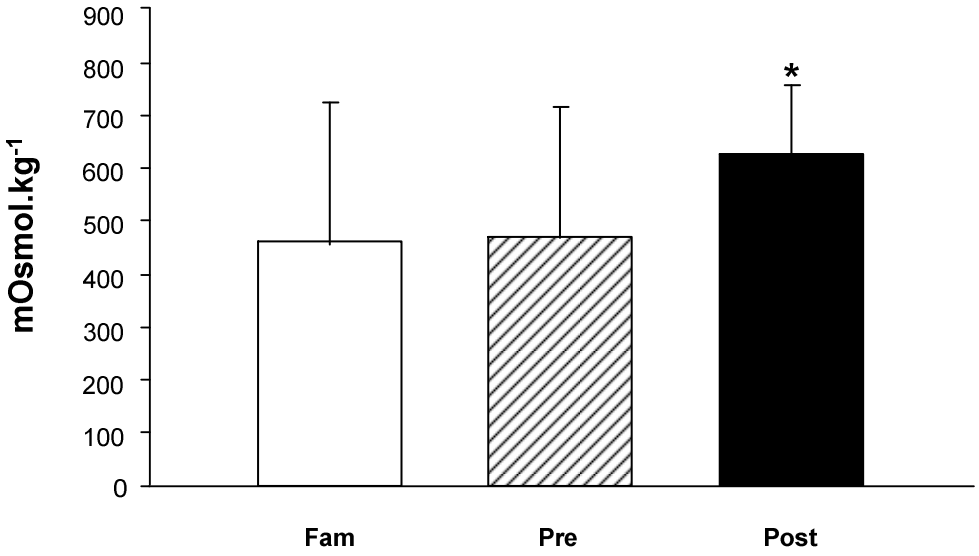

Figure 3.2. Change in mean Urine Osmolality (n=8). Comparisons between Familiarisation (Fam) Cr/Gly Pre-supplementation (Pre) and Cr/Gly Post-supplementation (post) Tests. Significant Change (*).

3.1.4. Haemoglobin (Hb) and Haematocrit (Hct)

Hb did not change significantly at anytime between familiarisation and pre-supplementation tests (P=0.974) (figure 3.3.). There were noticed reduction in Hb concentrations following Cr and Gly hyperhydration supplementation but it did not reach statistical significance when compared to pre-supplementation test (P=0.07). Hct did not change significantly at anytime between familiarisation and pre-supplementation tests (P=0.38) (figure 3.3.). However, after hyperhydration supplementation with Cr and Gly, there were significant reductions in Hct values when compared to pre-supplementation test (P<0.05).

There were significant decreases comparing the individual equivalent blood samples of pre-supplementation to post-supplementation tests (i.e. comparing the first blood sample of pre-supplementation test vs. the first blood sample of post-supplementation test and so on for the rest of the collected samples). In the 10 min blood sample, Hct showed high significant reduction by 1.26% ± 0.65% (P<0.05). In the 20 min blood sample, there was also a high significant decrease of Hct by 1.3% ± 0.96% (P<0.05). However, there was a higher significant change noticed in the 30 min blood sample comparing pre-supplementation and post-supplementation tests with a decrease in Hct by 1.36% ± 0.73% (P<0.05).

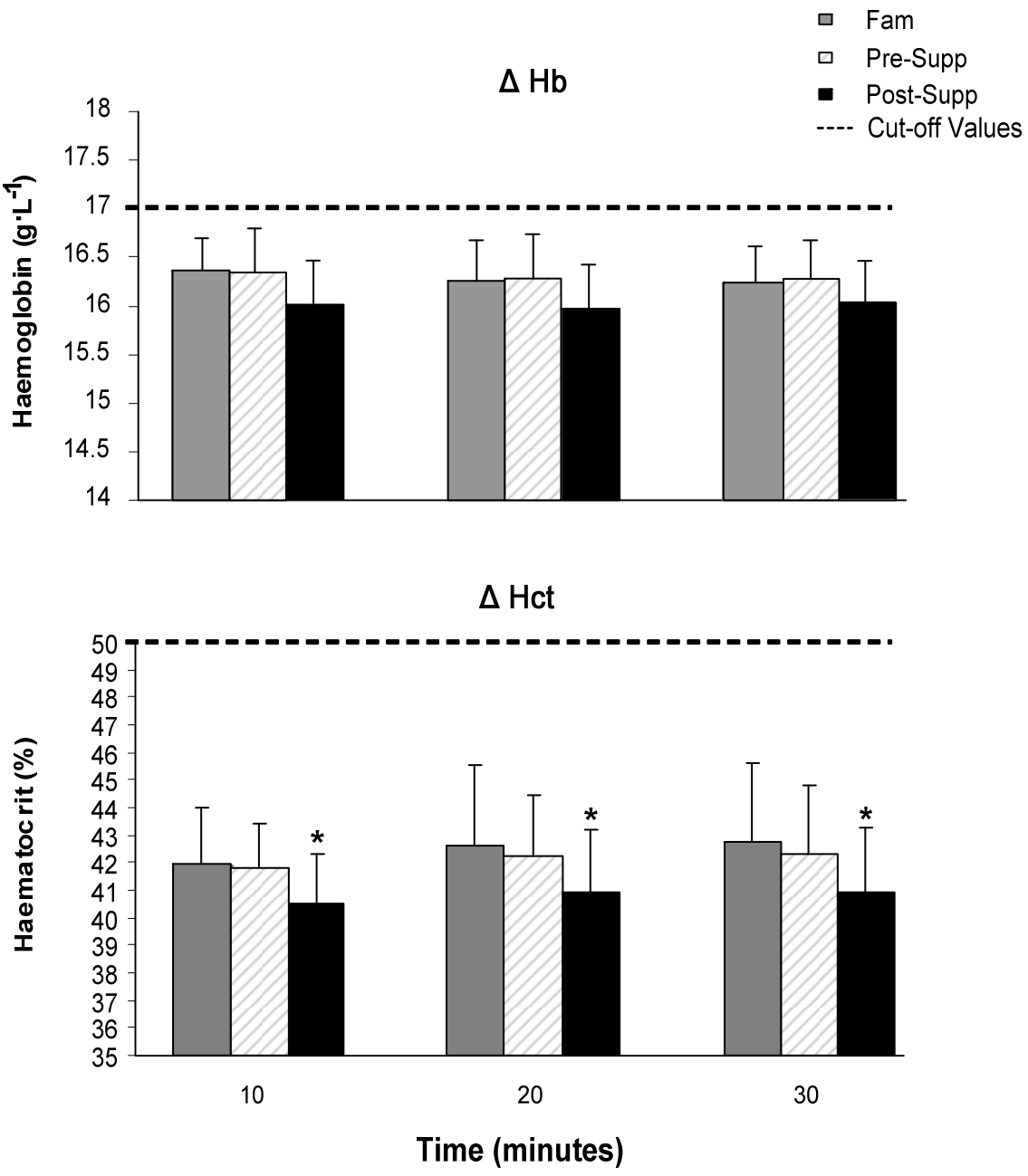

Figure 3.3. Change in mean Haemoglobin (Hb) and Haematocrit (Hct) (n=8). Comparisons between Familiarisation (Fam) Cr/Gly Pre-supplementation (Pre) and Cr/Gly Post-supplementation Tests (Post). Subjects' samples obtained at 10, 20 and 30 minutes of Supine Posture. WADA's and International Sports Federations Haematological Cut-Off Values (---). Significant Change (*).

3.2. Postural Changes Experiment (EXP2)

3.2.1. Haemoglobin (Hb) and Haematocrit (Hct)

Hb and Hct increased significantly ($P<0.05$) with the change of posture from supine to sitting and then to standing (figure 3.4.). Both Hb and Hct did not change significantly throughout the 30 min period of each individual posture ($P=0.09$ and $P=0.24$, respectively).

At the first 10 min blood sample, Hb concentration values increased significantly by 0.55 ± 0.06 $g.L^{-1}$ when subjects changed their posture from supine to sitting ($P<0.05$). Hb concentration increased significantly by 0.71 ± 0.14 $g.L^{-1}$ when posture was changed from sitting to standing ($P<0.05$). There was also a significant increase in Hb concentration by 1.27 ± 0.2 $g.L^{-1}$ comparing the values of the supine to standing posture ($P<0.05$). Hct values of each posture at the first 10 min blood sample showed a significant increase by $0.87\% \pm 0.12\%$ when subjects changed their posture from supine to sitting ($P<0.05$). Hct values showed significant increase by $1.70\% \pm 0.08\%$ when posture was changed from sitting to standing posture ($P<0.05$). There was also a significant increase in Hct by $2.57\% \pm 0.04\%$ comparing values of supine to standing posture ($P<0.05$).

In the second sample of each posture, i.e. 20 min blood samples, Hb increased significantly when subjects changed their posture from supine to sitting 0.85 ± 0.15 $g.L^{-1}$ ($P<0.05$). Hb also showed a significant increase when posture changed from sitting to standing by 0.76 ± 0.02 $g.L^{-1}$ ($P<0.05$). There was a significant change comparing the shift of supine to standing postures with increased values by 1.61 ± 0.16 $g.L^{-1}$ ($P<0.05$). Hct values of each posture at the 20 min blood sample increased significantly when posture was changed from supine to sitting $1.41\% \pm 0.25\%$ ($P<0.05$) and also increased significantly when posture changed from sitting to standing by $1.70\% \pm 0.3\%$ ($P<0.05$). There was a significant change in Hct comparing the shift of supine to standing postures with increased values by $3.12\% \pm 0.04\%$ ($P<0.05$).

In the third sample of each posture, i.e. 30 min blood samples, Hb increased significantly when posture changed from supine to sitting by 0.78 ± 0.36 $g.L^{-1}$

($P<0.05$) and when posture changed from sitting to standing, Hb changed significantly showing an increase by 0.94 ± 0.15 $g.L^{-1}$ ($P<0.05$). The increase of Hb was also significant when comparing the values of supine to standing posture 1.72 ± 0.37 $g.L^{-1}$ ($P<0.05$). Hct values of each posture at the 30 min blood sample increased significantly when posture changed from supine to sitting by 1.70% ± 0.21% ($P<0.05$) and when subjects changed their posture from sitting to standing Hct showed significantly increased values by 1.97% ± 0.28% ($P<0.05$). The increase of Hct was also very highly significant when comparing the values of supine to standing posture 3.67% ± 0.07% ($P<0.05$).

Looking at the individual data (figure 3.5.) none of the 10 subjects exceeded the 50% Hct cut-off whereas only 2 subjects exceeded the 17 $g.L^{-1}$ Hb cut-off at some point in one or more postures. The first subject's blood samples Hb exceeded the cut-off at the last 2 blood samples of the standing posture with values of 17.25 $g.L^{-1}$ and 17.75 $g.L^{-1}$, respectively. The second subject's blood samples exceeded the Hb cut-off at all postures. This subject's supine posture blood sample had Hb concentration of 17.2 $g.L^{-1}$ only at the 30 min sample. However, the second subject had the same reading of 17.1 $g.L^{-1}$ at the whole time course of the sitting posture. At the standing posture, this subject had exceeded the cut-off by the following Hb concentration values of 18.25 $g.L^{-1}$, 18.35 $g.L^{-1}$ and 18.2 $g.L^{-1}$, respectively.

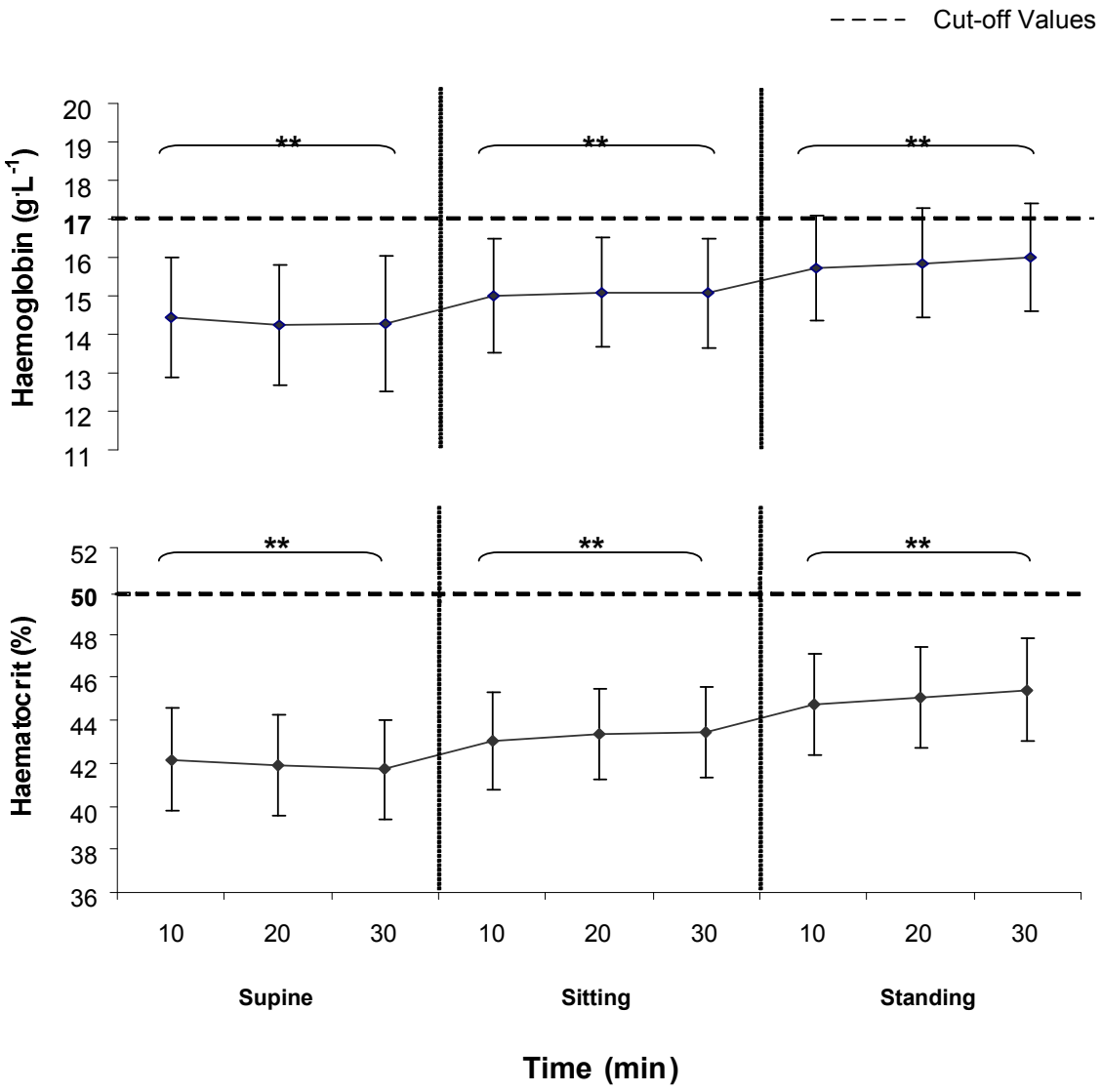

Figure 3.4. Change in mean Haemoglobine and Haematocrit (n=10). Comparisons between subjects' blood samples (Haemoglobin and Haematocrit) at Supine, Sitting and Standing Postures. Subjects' blood samples obtained at 10, 20 and 30 minutes of each posture. WADA's and International Sports Federations Haematological Cut-Off Values (---). Significant Change (**).

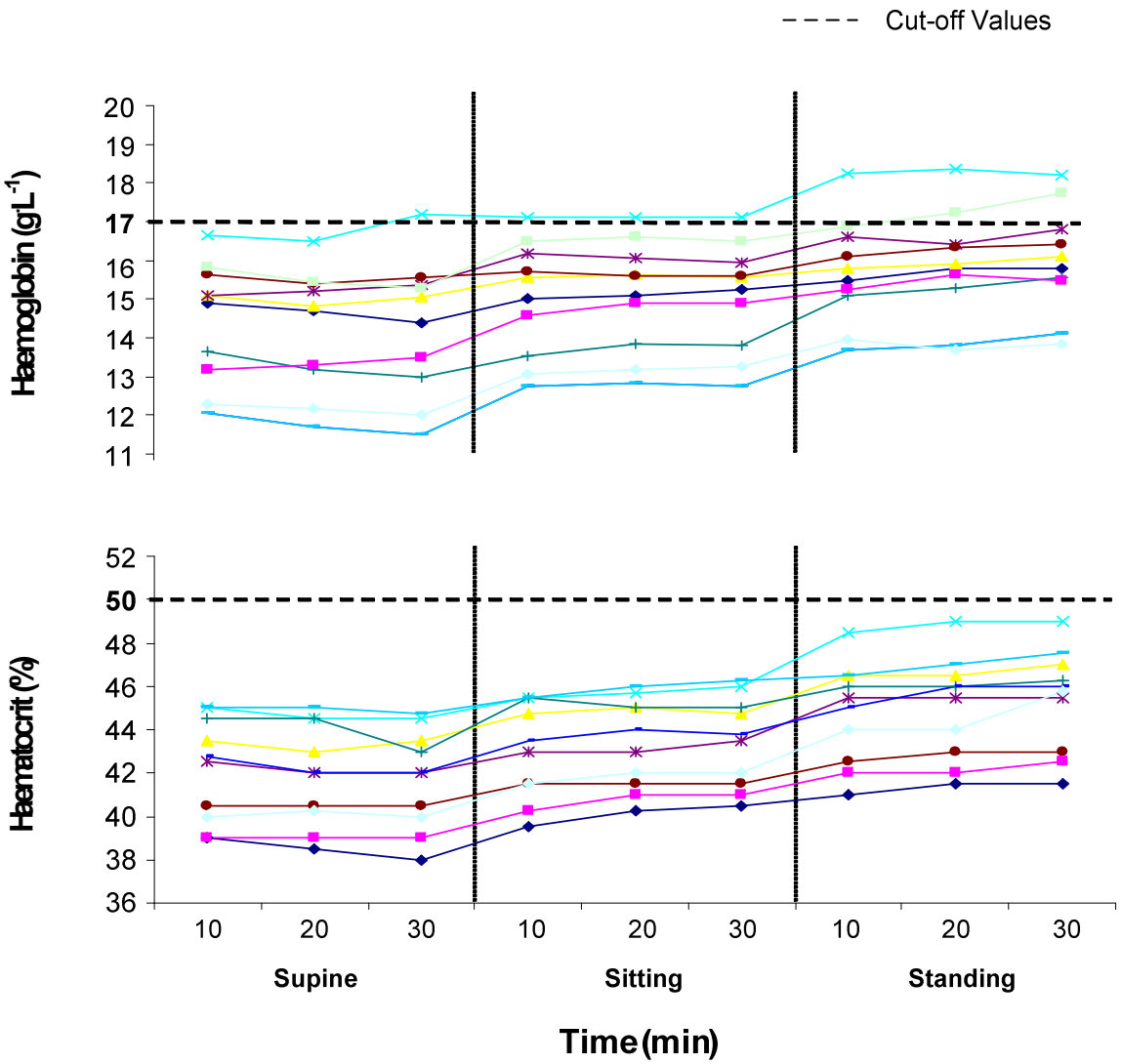

Figure 3.5. Change in individual Haemoglobin and Haematocrit values (n=10). Comparisons between individual subjects' blood samples (Haemoglobin and Haematocrit) at Supine, Sitting and Standing Postures. Subjects' blood samples obtained at 10, 20 and 30 minutes of each posture. WADA's and International Sports Federations Haematological Cut-Off Values (---).

3.3. Running with creatine and Glycerol Hyperhydration Experiment (EXP3)

3.3.1. Creatine Responders

All Subjects were responders to Cr supplementation with increased body mass ≥0.2 kg (Kilduff et al., 2003; Easton et al., 2007); except for one subject who only gained 0.15 kg after the supplementation trail.

3.3.2. Haemoglobin (Hb) and Haematocrit(Hct)

Hb and Hct did not change significantly at any point comparing all samples using general linear model with hyperhydration and time factors (P=0.74 and P=0.76, respectively) (figure 3.6.).

3.3.3. Reticulocytes (% Retics)

Reticulocytes (%Retics) did not change significantly at any point comparing all samples using general linear model with hyperhydration and time factors (P=0.72) (figure 3.7.).

3.3.4. OFF-hr Scores

OFF-hr Scores did not change significantly at any point comparing all samples using general linear model with hyperhydration and time factors (P=0.78) (figure 3.7.).

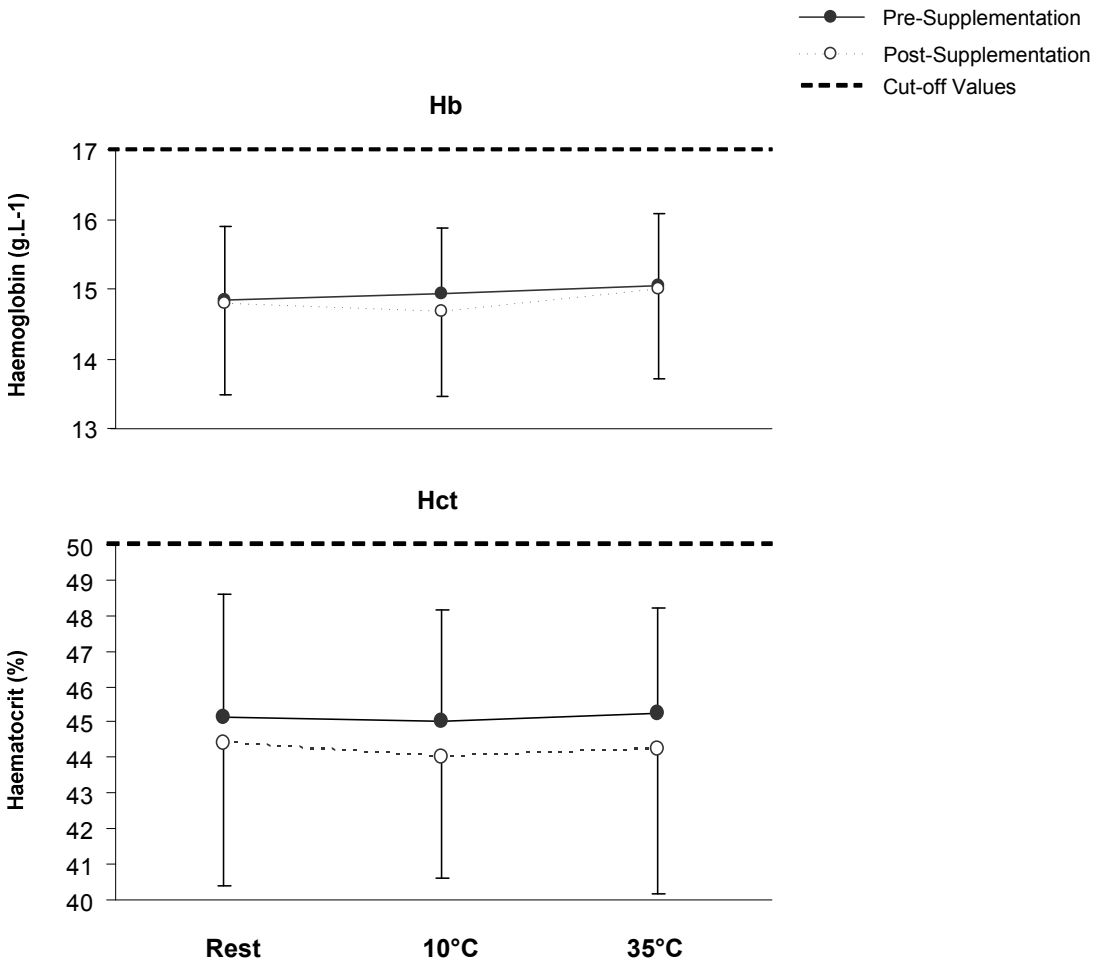

Figure 3.6. Change in mean Haemoglobin (Hb) and Haematocrit (Hct) before (—●— Pre-Supplementation) and after (○ Post-Supplementation) Cr/Gly Supplementation hyperhydration; at rest, post exercise at 10°C and post exercise at 35°C (n=7). WADA's and International Sports Federations Haematological Cut-Off Values (---). Athlete subjects' blood samples obtained at 10 minutes of Sitting Posture (WADA's Standards).

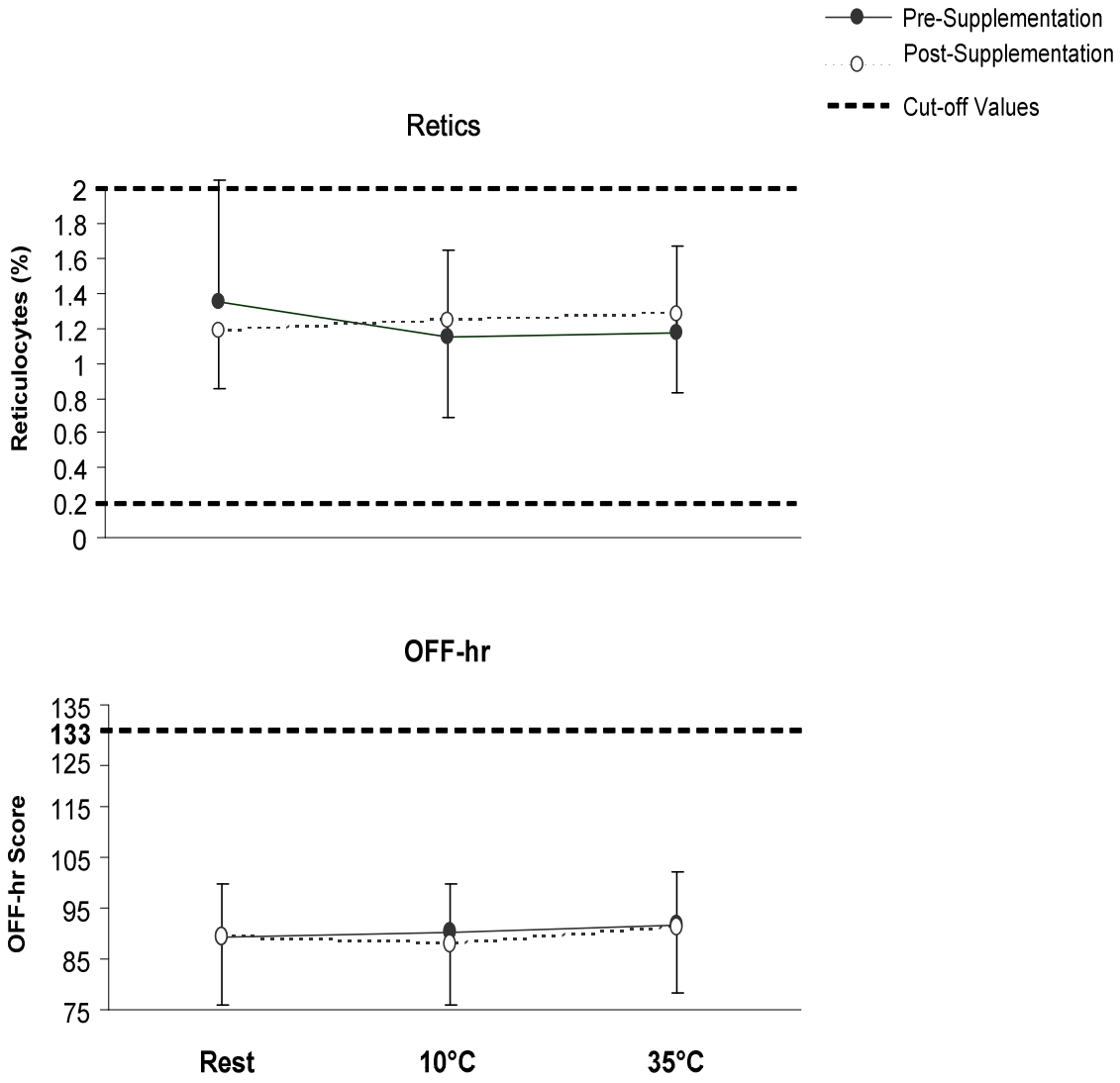

Figure 3.7. Change in mean Reticulocytes (Retics) and OFF-hr scores (2nd generation model from Gore et al., 2003) before (—●— Pre-Supplementation) and after (○ Post-Supplementation) Cr/Gly Supplementation hyperhydration; at rest, post exercise at 10˚C and post exercise at 35˚C (n=7). WADA's and International Sports Federations Haematological Cut-Off Values (---). Athlete subjects' blood samples obtained at 10 minutes of Sitting Posture (WADA's Standards).

CHAPTER 4

DISCUSSION

4. Discussion

4.1. General Discussion

The haematological cut-off values used in the three experiments accomplished in this thesis (i.e. EXP1, EXP2 and EXP3) refer to male subjects and athletes only and the discussion will be focused on male cut-off values and scores. The cut-off values of the haematological parameters and markers are used by some federations in collaboration of IOC and WADA to control and limit the abuse of prohibited substances and methods. This is prevalent in endurance sports and competitions where it is difficult to detect the doping substances like rHuEPO or doping methods like autologous blood transfusions. The first federation to apply the no-start rule was the UCI when blood doping was out of control and causalities and mortalities were witnessed in sports and competition fields among cyclists. For general health and safety reasons of the competing cyclists, the cut-off (highest) value of Hct was set at and not to exceed 50% for males and 47% for females. This cut-off value was especially set to avoid the complications of high blood viscosity of some elite cyclists during endurance cycling competitions, albeit a result of having naturally high Hct or elevated Hct by legal methods and substances (altitude training and supplements), or illegally by autologous blood transfusions and rHuEPO injections. However, WADA have not yet set any haematological cut-offs for “no- start” or “start prohibition” rules in the World Anti-Doping Code and the only purpose of blood testing for uses in blood doping substances are for blood substitutes, serum EPO and the ON model score of second generation model (Gore et al., 2003). The use of a positive ON model score of blood test acts as a pre-EPO urine test and confirmatory protocol due to the economically high costs and length of time spent to process and analyse urine samples.

Currently, some other haematological parameters cut-off values have been added and documented in some federations’ general practice and regulation documents and Anti-Doping rules with compliance to the World Anti-Doping Code. For example, Hct, Hb, %Retics and OFF-hr score of (Gore et al., 2003) second generation model and Hb_{Zscore} and $OFF\text{-}hr_{Zsore}$ the longitudinal haematological monitoring (Sharpe et al., 2006) third generation model. The

International Cycling Union (UCI) medical monitoring and medical tests results for sporting safety and conditions states in chapter 13 articles 13.1.062 and 13.1.63 that the athlete is considered unfit to compete and participate in UCI approved cycling events if Hct >50% with Hb >17 $g.L^{-1}$ for males and Hct >47% with Hb >16 $g.L^{-1}$ for females, OFF-hr score >133 for males and >123 for females and %Retics <0.2% and >2.4% for both males and females (UCI, 2009). For cyclists who have naturally high Hct values (50% for males and 47% for females), an international certificate should be issued and at least 4 blood tests within 6 weeks or 4 annual blood tests conducted. According to article 13.1.089, athletes who do not submit to the medical monitoring should provide a medical check up file from a haematologist and obtain a UCI certificate for naturally high Hct (UCI, 2009).

The international Ski Federation (FIS) did not consider the Hct in the FIS Anti-Doping Rules document and set the Cut-Offs for Hb, %Retics, OFF-hr score and with the addition of Hb_{Zscore} and OFF-hr_{Zsore}. If FIS registered and licensed athletes have the following: Hb ≥17 $g.L^{-1}$ for males and Hb ≥16 $g.L^{-1}$ for females, %Retics <0.2% and >2% for both males and females; and OFF-hr scores ≥125.6 for males and ≥113.5 for females and Hb_{Zscore} and OFF-hr_{Zsore} ≥3.09 then they are obliged to undergo "start prohibition" rule, according to FIS Anti-Doping Rule article FIS B.4.1 to B.4.3, if they (FIS, 2009).

The International Associations of Athletics Federations (IAAF) has set the Hct, Hb and %Retics and OFF-hr scores cut-offs according to the previous scientific research and similar to that of UCI cut-offs but without direct "start prohibition" rule. The reason for not applying the "start prohibition" or "no-start" rules might be because of the naturally and abnormally high blood profiles of certain leading champion athletes with consistent dominance in endurance and long distant running, specifically East African runners (Ojiambo et al., 2008; Moore et al., 2007; Parisotto et al., 2000b). The cut-offs applied are as follows: Hct >50% for males and Hct >47% for females, Hb >17 $g.L^{-1}$ for males and Hb >16 $g.L^{-1}$ for females, % Retics <0.2 % and >2.0 % for both males and females and OFF-hr >133 for males and >123 for females. If these are exceeded, the athlete should provide a urine sample for screening of prohibited substances (IAAF, 2009; Ojiambo et al., 2008; Moore et al., 2007) before banning, or apply the "no-

start" rule to their athletes which follow the WADA's standard rules for banning athletes and act like a pre-EPO urine test as aforementioned (WADA, 2001; WADA, 2009b). WADA is currently considering the abnormal haematological profiles and cut-offs of OFF-hr sores (Gore et al., 2003) and Hb_{Zscore} and OFF-hr_{Zsore} values (Sharpe et al., 2006) as stated by Ojiambo et al. (2008).

Previous studies conducted in our laboratories showing the synergistic effects of the hyperhydration induced by ingesting Cr and Gly (Easton et al., 2007; Easton et al., 2006) succeeded in increasing subjects' plasma volume. The same fluid loading protocol has been replicated in EXP1 except for the time and posture at which blood samples were withdrawn. Easton et al., (2007) obtained blood samples every 10 min for 40 min from the subjects whilst seated on cycle ergometer and Easton et al., (2006) obtained blood from the subjects after 15 min of seated position. The authors of both studies obtained the blood samples from a superficial vein located on the dorsal surface of the hand while in EXP1 the blood was withdrawn from the anticubital vein as done by previous leading research on blood and EPO doping (Parisotto et al., 2001; Sharpe et al., 2002; Ashenden et al., 2003). Easton et al., (2007) and Easton et al., (2006) used the same methods to calculate plasma volume changes from the changes in Hct and Hb (Dill and Costill, 1974) of pre-supplementation trials in relation to post-supplementation trials with the assumption that there were no changes in RBCs during the seven days of Cr and Gly supplementation period. Therefore, the decreases in Hct and Hb values found in EXP1 were the consequence of hyperhydration which was generated by supplementation of Cr and Gly for 7 days representing the reductions in plasma volume and vice versa (Easton et al., 2009).

Body mass increase (figure 3.1.) in EXP1 represents water retention and as indirect measurement of such retention induced by the ingestion of Cr and Gly (Easton et al., 2007). TBW increased significantly in EXP1 (figure 3.1.) after Cr and Gly supplementation for 7 days by 0.67 ± 0.17 L ($P<0.05$). The addition of Gly to Cr supplementation for 7 days in EPX1 may overcome the phenomenon of having non-responders to Cr as all subjects have gains of more than 0.2 kg body mass compared to a previous study in which Cr non-responders were reported (Kilduff et al., 2003). It is well known that Gly increases ECW (Nelson and

Robergs, 2007; Koulmann et al., 2000) while Cr raises ICW (Kilduff et al., 2004). However Cr played the major role in this fluid retention process as the ICW was greater than ECW which was induced by the effect Gly ingestion (figure 3.1.).

In EXP1 (figure 3.3.), Hct values in all 3 blood samples during the 30 min supine laying were decreased significantly by the effects of the induced hyperhydration of Cr and Gly ingestion, whilst there were no significant reductions in Hb concentration ($P=0.07$). Hb is located within RBCs and considered as an intracellular molecule. Theoretically any increases in ICW compartment should affect the concentration of its content including Hb molecules concentration (Easton et al., 2007; Dill and Costill, 1974; Nelson and Robergs, 2007). However, this was not the case in EXP1 even when the major increase in TBW influenced the raise in ICW. In contrast, Hct, which is known as pack cell volume and represents the percentage RBCs in whole blood that is located within the vessels and considered as an extracellular fluid (Easton et al., 2007; Dill and Costill, 1974; Nelson and Robergs, 2007), was highly affected by such hyperhydration and the increase in ECW which was considered minor compared to the water retained within ICW.

The 10 min blood sample results from EXP1, showed a significant reduction by 1.26% ± 0.65% ($P<0.05$) in Hct. With the progression of time at the supine posture of EXP1 (figure 3.3.), the 30 min blood sample showed a greater reduction of Hct values of 1.35% ± 0.73% ($P<0.05$) proving the effects of prolonged supine posture and hyperhydration on haemodilution. The supine posture of EXP1, in which blood samples were withdrawn, has been previously used by leading research studies on EPO and blood doping in the process of inventing the first and second generation models to detect such prohibited substances and methods but the samples were taken at 5 min and did not exceed 10 min. (Parisotto et al., 2000a; Parisotto et al., 2001; Ashenden et al., 2001; Sharpe et al., 2002; Ashenden et al., 2003). The authors of these studies emphasised the effects of prolonged supine posture on blood parameters and markers of blood doping. These reduced Hct values in all 3 blood samples of EXP1 suggest may result in false negatives of athletes' blood samples. Particularly, endurance athletes with abnormally and illegally Hct values equal or slightly higher than the 50% Hct cut-off value can abuse such hyperhydration

and posture protocols when being tested on the year round monitoring program, to create haematological passports (out-, pre- and in competitions), where "start prohibition" or "no-start" rules are applied by federations. For athletes with naturally high Hct values, this hyperhydration protocol of EXP1 could be used as a safe method in preventing high blood viscosity which can occur during prolonged exercise and endurance events.

Gly and Cr ingestion has been found to reduce excreted urine volume and output (Anderson et al., 2001; Hultman et al., 1996). Urine osmolality in EXP1 (figure 3.2.) increased significantly by supplementing with Cr and Gly (632 ± 137 $mOsmol.kg^{-1}$; $P<0.05$). This increased osmolality is very effective and beneficial for selected tested athletes prior to providing urine sample. Diluted urine samples are rejected or suspended and the athletes are not allowed to leave the doping station without providing a urine sample of the required concentration. As stated by WADA in the Guidelines for Urine Sample Collection; article 5.11.11 that ***"The DCO (Doping Control Officer) shall confirm that the sample meets the requirements for analysis, as specified by the ADO (Administrative Doping officer) in accordance with the laboratory standards, by testing the residual volume of urine remaining in the collection vessel for specific gravity (greater than or equal to 1.005 if using a refractometer, or 1.010 with lab sticks, or as specified by the relevant laboratory) and, if necessary, pH (between 5 and 7, or as specified by the relevant laboratory). Reagent strips and/or a refractometer may be used."*** (Guidelines for Urine Sample Collection WADA, 2004; World Anti-Doping Code WADA, 2009b). This supplementation protocol in EXP1 has such an advantage over other hyperhydrating drinks that may result in decreased water retention and diluted urine sample. The time after notifying the athlete to arrive to the Doping Control Station and provide a urine sample ranges between 30 min to 60 min especially for in-competition samples (Guidelines for Urine Sample Collection WADA, 2004; World Anti-Doping Code WADA, 2009b). Athletes, especially endurance athletes, delaying to provide their urine samples for any reason is very favorable in preventing false positives of r-HuEPO doping (Beullens et al., 2006). Doing such a strategy accompanied by hyperhydration protocol used in EXP1 can ensure the time to provide concentrated urine sample and avoidance of suspicion of r-HuEPO abuse, which may result in suspensions and penalties.

These strategies are not to encourage blood doping but to help clean athletes form some of the complications occurring in the Doping Control Stations in an attempt to provide a prompt urine sample according to WADA standards.

The increase in muscular ICW is induced by Cr loading supplementation due to the ability of Cr to create an osmotic gradient within the cells that draws the water from the extracellular compartment and intercellular space into the intracellular compartment (Kilduff et al., 2003; Kilduff et al., 2002; Mesa et al., 2002). The decrease in the excreted urinary volume is also associated with Cr supplementation especially in the initial days of Cr ingestion (Hultman et al., 1996). This water retention was only considered an adverse side effect when Cr supplementation was used only as an ergogenic aid in sports with high and maximal intensity nature of exercise due to the accompanied increase in body mass (Bemben and Lamont, 2005). However, water retention and hyperhydration effects of Cr ingestion have been used and are currently being applied in many situations for positive gain. For example, endurance type sports, prolonged exercise and different environmental situations that have certain homeostatic effects on the athletes hydration status (Easton et al., 2007; Kilduff et al., 2004; Hadjicharalambous et al., 2008; Vogel et al., 2000) and in other medical purposes like the prevention of orthostatic intolerance and possible treatment of pre-syncope and syncope (Easton et al., 2006; Easton et al., 2009).

Hyperhydration and water retention induced by Cr supplementation can affect plasma volume. Sixteen subjects were supplemented with either Cr or placebo (Vogel et al., 2000) prior to cycling on a cycle ergometer for 160 minutes (at 32°C with 50% relative humidity) totally to induce a hypohydration status. Significant reductions were found in plasma volume in both groups, with higher losses in the placebo group (-9%) than Cr group (-7%) as well as losses in body mass which were higher in placebo group (-4%) than Cr group (-2.5%) compared to pre-test plasma volume and body mass (Vogel et al., 2000). In contrast, there were no significant increases in plasma volume in 21 subjects between placebo and Cr group in a study by Hadjicharalambous et al. (2008) with similar Cr supplementation and exercise conditions as Vogel et al.(2000) which included 20 $g.day^{-1}$ + 140 $g.day^{-1}$ glucose for 7 days on cycle ergometer for 40 min at temperature of ≈30°C with ≈70% relative humidity (Hadjicharalambous et al.,

2008). Although, there were no changes in plasma volume between the 2 groups post-supplementation TBW and body mass were significantly higher in Cr group as well as there being a tendency for ICW to be higher, although this did not reach statistical significance (Hadjicharalambous et al., 2008). Easton et al. 2007 tested 24 subjects with a similar Cr loading dosage, environmental conditions and length of exercise with the addition of Gly supplementation groups (Easton et al., 2007) to the above studies with 4 combinations of supplementation groups as follows: placebo/placebo, Placebo/Gly, Cr/Placebo and Cr/Gly. Plasma volume did not significantly change following supplementation and post-exercise in any groups. There were significant hyperhydration and increases in body mass, TBW, ICW and ECW in all groups except for placebo/placebo group and ECW in Cr/Placebo group (Easton et al., 2007). The latter finding emphasises the effect of Cr supplementation on increasing ICW with no influence on ECW.

Cr supplementation has been shown to influence body mass and TBW via increasing ICW. However, few factors may disrupt this response. The first factor is how well Cr is dissolved in the supplement fluid. The second factor is the intake of caffeine (Vandenberghe et al., 1996) and other diuretics on the lost retained water in urine. All the subjects of EXP1, EXP2 and EXP3 refrained from consuming caffeinated drinks in the test days. The third factor is how Cr individual respond positively or neutrally to Cr supplementation in the intramuscular uptake of ingested Cr which follows certain criteria Greenhaff et al. was the first to categorise Cr supplemented subjects into “responders” and “non-responders” (Greenhaff et al., 1994). Muscle biopsies were obtained from 8 subjects’s quadriceps muscle and precisely vastaus lateralis after isometric exercise pre- and post-supplementation with Cr at a dose of 20 $g.day^{-1}$ for 5 days. Muscle PCn, free Cr and total Cr (PCn +free Cr) were measured from the freeze dried biopsies as $mmol.kg^{-1}$ dry matter. From the results of the concentration of total muscle Cr, subjects were then categorised as “responders” (total Cr $\leq$120 $mmol.kg^{-1}$ dry matter) and “non-responders” (total Cr $\geq$120 $mmol.kg^{-1}$ dry matter). The responders had lower pre-supplementation total Cr and better muscular PCr resynthesis and Cr uptake during recovery periods after exercise bouts (Greenhaff et al., 1994). Kilduff et al. used similar criteria but the total intramuscular Cr was calculated from urine samples of 32

subjects which were estimated and corrected for the excreted creatinine in urine (Kilduff et al., 2002). Total intramuscular Cr uptake was calculated from subtracting the supplemented Cr daily dosage from urinary excreted Cr which was creatinine corrected. The subjects were categorised as "responders" (total Cr ≥32 mmol.kg^{-1}.dry weight muscle) and "non-responders" (total Cr ≤21 mmol.kg^{-1}.dry weight muscle) after 5 days of Cr supplementation (20 g.day^{-1} +180 g.day^{-1} dextrose) compared to placebo group (200 g.day^{-1} dextrose) followed by isometric exercise. The results showed increased fat free mass, body weight, TBW, peak and total force in the Cr responders compared to placebo group (Kilduff et al., 2002). In another study by Kilduff et al., the same method was used to estimate the total intramuscular Cr uptake (Kilduff et al., 2003). However, another criterion was added in this to the phenomenon of Cr supplementation responder subjects with optimal intramuscular uptake. Subjects were considered Cr "responders" if they gained >0.2 kg compared to their pre-supplementation body mass (Kilduff et al., 2003). In this study, Cr was supplemented over 4 weeks period and compared placebo and total Cr was calculated before and after isokinetic and isometric exercises in 19 subjects. Placebo and Cr supplemented groups underwent two distinctive loading and maintenance phases which consisted of 7 and 21 days respectively. In the loading phase, Cr was supplemented at a dose of 20 g.day^{-1} Cr + 140 g.day^{-1} glucose whereas placebo was supplemented as 160 g.day^{-1} glucose. The maintenance phase started from the eighth day until the end of the four weeks in which Cr group was supplemented with 5 g.day^{-1} Cr + 35 g.day^{-1} glucose and placebo group ingested 40 g.day^{-1} glucose. The results showed significant ergogenic and hyperhydration improvements in the "responders" who had greatest body mass increases >0.2 kg associated with the highest estimated intramuscular Cr uptake values (Kilduff et al., 2003). The improvements in hyperhydration status due to water retention (caused by Cr supplementation) was the highest in the "responders" which resulted in dramatic significant increases in TBW that was affected by the significant increases in ICW (intracellular water retention) without significant effects on ECW condition (Kilduff et al., 2003). This notion of differentiating subjects into Cr supplementation "responders" from "non-responders" were also used in another published study that was based on post-supplementation body mass increases of

>0.2 kg (Easton et al., 2007). In EXP1, all the 8 subjects were responders to Cr with gains over 0.2kg.

On the other hand, Gly influences the increases in ECW more than ICW (Easton et al., 2007). Gly acts more efficiently as a hyperhydating agent and supplement for its hyperosmotic properties and its distribution among all fluid compartments which is enhanced when accompanied with further ingestion of water volumes (Nelson and Robergs, 2007). Furthermore, the direct effects of Gly on the kidneys to reabsorb the excess water from the circulation via raising the renal medullary concentration (osmotic) gradient is the actual mechanism independent of hormonal reactions and responses by which Gly works, produces and induces measured hyperhydration (Freund et al., 1995; Melin et al., 2002). Gly is primarily reabsorbed through the renal proximal convoluted tubule via passive diffusion (Kruhoffer and Nissen, 1963) and may also have a contribution from active transport (McCurdy et al., 1966). The hormonal mechanisms, responses and reactions of the antidiuretic hormone (ADH), aldosterone and natriuretic peptide (ANP) that induce renal water reabsorption is not affected and can not be applied with Gly ingestion (Freund et al., 1995). Melin et al. also tested the hormonal and renal responses of the hyperhydration induced by 1.1 g.kg $^{-1}$ of body weight of Gly supplementation which was mixed with fluid in eight healthy subjects and also concluded that there were no significant changes in the concentration of plasma ADH, ANP, rennin and aldosterone (Melin et al., 2002). In addition, these authors also stated that renal responses to retain fluids and induce hyperhydration (assessed by bioelectrical impedance) is by the effects of Gly ingestion rather than these fluid-regulating hormones (Melin et al., 2002). Furthermore, Gly ingestion with additional fluids intake decreased the rate by which free water is cleared from the circulation (Freund et al., 1995). Gly turnover (Bortz et al., 1972) plays a major role in offsetting the efficiency of the gained hyperhydration and fluid retention which provides the need for regular supplementation of periodic and regular Gly doses to maintain that hyperhydration status (Bortz et al., 1972; Nelson and Robergs, 2007). The regular supplementation of Gly at a dose of 1.0 g.kg $^{-1}$ body mass twice daily (once in the morning and once in the evening) showed significant gains and increases plasma volume which was explained by the increase in TBW and specifically in the ECW compartment (Easton et al., 2006; Easton et al., 2007).

These findings supports the results of EXP1 that leaded to decreased Hct and Hb compared to the results of EXP3 which will be discussed shortly.

The significant change in Hb and Hct ($P<0.05$) was noticed upon alternating the posture from supine to sitting and ending with standing which was influenced by the progression in time of each posture in EXP2 (figure 3.4.). However, there was no recorded change of Hb and Hct between the 3 blood samples within each individual posture ($P=0.09$ and $P=0.24$, respectively). The standing posture in EXP2 served as a control posture for both the supine and sitting postures. The Hb and Hct values of the standing posture were significantly ($P<0.05$) higher than the supine and sitting postures. The standing posture causes the blood to pool distally in the lower limbs by the effects of gravitational pull and the limited venous return to lower limbs. Venous return can be enhanced by increasing muscular contraction. In EXP2, the subjects were advised to minimise the calf muscles contractions, ankles and feet movements unless they were very uncomfortable. Similar results of increased Hb and Hct and decreased plasma volume were found with 70° and 60° standing in tilt table (head up tilt) experiments (Easton et al., 2009; Qi et al., 2005). This standing posture in EXP2 resulted in higher values of Hb and Hct at the 30 min blood sample compared to the 20 min and 10 min blood samples.

Throughout all the supine posture period of EXP2 (figure 3.4.), Hb and Hct values were decreasing with the progression of time but again did not change significantly when comparing these parameters of the 30 min blood sample to the 10 min blood sample. In contrast, Hb and Hct values of the sitting posture were increasing with the elapse of time and results were highest in the 30 min blood sample compared to the 10 min blood sample and this change was also insignificant. These dissimilar physiological responses upon the duration and time spent in each posture is mainly explained by partial blood pooling in the lower limbs in the sitting posture and the eliminated effects of the gravity forces on blood vessels and the functional and structural anatomy of the human body. The minimum significant increases in Hb and Hct noticed in sitting posture compared to equivalent blood sample (i.e. 10 min sample) of the supine posture were 0.55 ± 0.06 $g.L^{-1}$ and $0.87\% \pm 0.12\%$, respectively ($P<0.05$). This raise in Hb and Hct shows that supine posture has a haemodiluting effect and potential to

expand plasma volume. Therefore, WADA's only approved posture and time which are sitting and withdrawal of blood at 10 min are very acceptable. In addition, venipuncture in this posture is more visible to the selected athlete and his/her representative is more legally accepted according to WADA's World Anti-Doping Code and the guidelines of blood sample collection.

Focusing on the individual Hb and Hct values of each subject (figure 3.5.), no subject exceeded the 50% Hct cut-off whereas only 2 subjects exceeded the 17 $g.L^{-1}$ Hb cut-off at some point in one or more postures. This implicates that athletes having borderline Hb concentrations (naturally or legally) close to the Hb cut-off of blood samples withdrawn at supine posture may possibly be included in suspicious lists or prohibited from competing in official sporting events where blood doping tests are applied by following WADA's guidelines (i.e. sitting posture for 10 min). EXP2 shows that postural changes from supine (0°) to sitting and ending with standing posture has more effect and impression on Hb concentration than Hct% values. However, to make such a conclusion, endurance athlete subjects, possibly with higher Hb and Hct values and different competition disciplines, should be recruited with the same protocol and design of EXP2 and with the inclusion of more blood parameters and markers that may be affected by endurance performance and blood doping.

The hyperhydration protocol in EXP3 differed slightly from EXP1 in which Gly was ingested at the test day and precisely 5 hours prior to the first blood sample. The addition of Gly to Cr at the seventh day of fluid loading protocol was to reduce the feeling of stomach fullness and the tendency to feel nauseous among the selected trained subjects in EXP3. All subjects in experiment 3 were actively involved in exercise training (some competed in local sporting competitions). Using Gly for 7 consecutive days may delay their training programs caused by delayed stomach emptying and fullness. The posture and time, which were sitting and waiting for 10 min prior to blood sample withdrawal, of EXP3 were modified from EXP1 to match WADA's only approved posture and time (WADA Standards and Harmonization Guidelines for Blood Collection WADA, 2008). The addition of 2 exercise bouts of 30 min running once in slightly cool damp temperature (10°C with 70% relative humidity) and the other in warm humid temperature (35°C with 70% relative humidity) in an environmental chamber at

60% of their VO_2 max to mimic the physiological effects of the changes of temperatures and endurance work exerted, with regard to time frame, in real exercise field on blood markers before and after hyperhydration.

Gly absorption and emptying from gastrointestinal tract is usually delayed, causing an acute dehydration explained by lower plasma volume for the shift of fluids from the plasma contained in the circulating blood to gastrointestinal tract where Gly is located (Easton et al., 2006; Freund et al., 1995). However, this mechanism is transient and the duration depends on the concentration and dose of Gly and the volume of fluid ingested with it. The speed by which the gastrointestinal tract empties Gly is equivocal and still debatable that leaded to many questioning whether Gly is effective as a hyperhydrating agent and supplement. However, much of the varying results found in the literature may be explained by the different Gly hyperhydration protocols (e.g. with water or without), methods (e.g. before, after or without exercise) and concentrations which have been used. However, much of the work done in our laboratories using different timings of Gly ingestion protocols (e.g. 1 hour, 3 hours and 5 hours) before testing (Easton et al., 2006) has overcome these methodological issues and concluded that it takes 5 hours for Gly to be totally absorbed and distributed among body fluid compartments (especially ECW) to induce hyperhydration (Easton et al., 2007). The use of Gly supplementation with exercise in hot environment have also been tested with the last dose (1.0 $g.kg^{-1}$ body mass) of Gly consumed with the addition of fluid 5 hours prior to testing (Easton et al., 2007).

The hyperhydration protocol and blood withdrawal posture before and after running in different temperatures used in EXP3 resulted in insignificant changes of 4 measured and calculated blood markers: Hb, Hct, %Retics and OFF-hr comparing pre-supplementation to post-supplementation tests (figures 3.6. and 3.7.). These results are thought to be by the influence of a single Gly bolus ingestion rather than for 7 days as in EXP1. Among these parameters, Hct had the highest tendency and sensitivity to decrease in EXP3 hyperhydration protocol and posture. Hb, %Retics and OFF-hr scores were not affected by EXP3 hyperhydration and posture protocols. In addition, %Retics post-supplementation values at rest were lower at rest and increased after both running bouts

compared to pre-supplementation. To prevent statistical errors, these statistical results and analyses were performed with and without the inclusion of a non-responder subject to Cr supplementation who only gained 0.15 kg after the supplementation protocol; as mentioned earlier responders to Cr loading supplementation are characterised by the increase in body mass ≥0.2 kg (Easton et al., 2007; Kilduff et al., 2003). However, no significant changes in Hb, Hct, %Retics and OFF-hr score were noticed by running the statistical analysis in either ways of including and excluding this non-responder subject's data.

Lyons et al. used Gly only to hyperhydrate 6 subjects with 1.0 g.kg $^{-1}$ body mass 3 times within 4 hours with large fluid intake prior to 90 min exercise bout separated by 5 min rest at every 30 min interval in hot dray environment (42°C with 25% relative humidity) at 60% of subject's VO_2 max. The blood samples were withdrawn from the subjects in a sitting posture and Hb and Hct values showed no significant changes following Gly ingestion (Lyons et al., 1990). These results are in agreement of EXP1 hyperhydration loading protocol that contradicts the effects of Gly ingestion on the test day only as in EXP3. Easton et al. (2007) used the Cr and Gly hyperhydration for 7 days with fluid loading followed by cycling exercise bout in warm humid environment (30°C with 70% relative humidity) and the blood was withdrawn while subjects had seated for 6 minutes prior to the start of the endurance cycling test and ending with 40 min blood sample in same posture at the completion of the exercise bout. This supplementation for 7 days resulted in significant increases in body mass, TBW, ECW, ICW, serum osmolality with the attenuation of core temperature but did not succeed in expanding the plasma volume significantly (Easton et al., 2007).

Upon the development of the first generation blood doping detection model the authors pointed out that Hct is an unreliable blood parameter and can be easily manipulated by postural effects and saline infusions (Parisotto et al., 2000a). This phenomenon of decreased and diluted Hct values was demonstrated and explained in EXP1, EXP2 and insignificantly in EXP3. UCI combined the 50% Hct value with the 17 g .L^{-1} concentration cut-off for males instead of Hct alone to apply the "no-start" rule (UCI, 2009). Furthermore, FIS excluded Hct values from the "start prohibition" rules (FIS, 2009). Upon the development and establishment of the second generation blood doping detection model equations,

Hct was not included in any of the four ON and OFF model equations (Gore et al., 2003). Likewise, the third generation blood doping detection model equations included mean longitudinal Hb concentrations and OFF-hr scores known as Z scores without any regard to Hct values (Sharpe et al., 2006). In EXP3, none of Hb, %Retics or OFF-hr scores were affected by the supplementation protocol when blood was withdrawn from subjects resting in sitting posture for 10 min before or after exercise and did not result in haemodilution.

These findings emphasise the need for Gly to be ingested with Cr for the whole 7 days of fluid hyperhydration loading period (as in EXP1) and not only on the competition or test day (as in EXP3). The same protocol of Easton et al. (2007) was duplicated very recently in our laboratories and showed body mass gains up to 1.7 kg that represents increases in TBW. This suggests the use of both Cr and Gly in addition to carbohydrates for 7 days should be implemented in future hyperhydration research aiming to increase TBW and expanding plasma volume. In addition, it is always important to keep in mind the WADA's standards in which the blood sampling should be in sitting posture for 10 min prior to venipuncture.

4.2. Conclusion

The hyperhydration protocol of EXP1 using Cr and Gly for 7 days succeeded in significantly manipulating and increasing body mass, TBW, ECW, ICW and urine osmolality and decreasing Hct values but not Hb concentration when blood was withdrawn in supine (0°) posture. In EXP2, the change of posture from supine (0°), to sitting and ending with standing resulted in haemoconcentration and increases in Hct and Hb values. When the approved posture of WADA, which is sitting for 10 min prior to blood sample withdrawal from subjects was applied in EXP3 with a slight change in hyperhydration protocol of Cr for 7 days and Gly on the seventh day only, it resulted in no significant changes in Hct, Hb, %Retics and OFF-hr scores and therefore no haemodilution effects when compared to pre-supplementation tests at rest and following submaximal exercise bouts in cool and warm environments. The hyperhydration protocol of EXP1 was more successful in decreasing Hct and Hb than EXP3. This was influenced by the

ingestion of a single bolus of Gly on the test day in EXP3 rather than a loading period of 7 days of Cr/Gly supplementation in EXP1 (fluid loading drink made up to 1 L twice daily for 6 days and once 5 hours prior to the first blood sample of the supplementation test day). In all three experiments (EXP1, EXP2 and EXP3), Hct among other blood parameters was the most affected by hyperhydration, posture and time at which blood samples were obtained. However, Hct is not a critical blood parameter in detecting and deterring blood doping in leading research or been published as such in peer-reviewed journals, nor has it been introduced into WADA's standards or adopted by federations with endurance athletes. Moreover, the standardisation of blood and urine samples obtaining protocols of posture, time, altitudes and analytical methods play a decisive role in which substances or a methods are considered be legal, or not, in the research area of doping in general. Upon the completion of these experiments (publication of the Easton et al., 2009 manuscript) and presentation to WADA, Gly was added to WADA's prohibited substances list (The 2010 Prohibited List International standards, WADA, 2010; Easton et al., 2009).

More research is required to recruit more endurance athletes with hyperhydration using Cr and Gly with fluid loading for 7 days at sea level as well as at altitude with WADA's approved posture and time of blood sample withdrawal. The addition of glucose to the Gly and Cr mix should be considered as it has been used in previous studies done within our laboratories (Easton et al., 2006; Easton et al., 2007) and recently unpublished work from our laboratories that has resulted in substantial increases in body mass and provide hyperhydration results.

More research is suggested to focus on markers and parameters that are affected by blood doping methods and substances which are very difficult to detect without the incidences of false positives like autologous blood transfusions and r-HuEPO injections and which are not susceptible to dilution and manipulation by masking methods and substances. Such markers could be certain genes that can be monitored and studied when they are activated or inhibited (switched on and off) by the use of these illegal blood doping substances and methods or other legal scientific proven and WADA allowed methods like living or training at altitude.

Appendix

Dr Yannis Pitsiladis
Reader in Exercise Physiology and Director of ICEARS
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University of Glasgow
G12 8QQ

UNIVERSITY of GLASGOW

24/04/2006

Dear Dr Pitsiladis

FBLS Ethics Committee for Non Clinical Research Involving Human Subjects: FBLS 0614 - The effects of combined creatine and glycerol hyperhydration on cardiovascular responses to postural change.

With regard to the above-named application which you recently submitted to the FBLS Ethics Committee for consideration, I am pleased to let you know that the Committee has given its approval without qualification, for the period 24th April 2006 to 30th September 2007. Please advise the Committee if this time scale is exceeded. I would be grateful if you would provide the committee a short report on the project by 31st December 2007.

As a condition of approval and in linc with thc committcc's need to monitor research, the following requirement applies: any unforeseen events which might affect the ethical conduct of the research, or which might provide grounds for discontinuing the study, must be reported immediately in writing to the ethics committee from which you have received approval. The committee will examine the circumstances and advise you of its decision, which may include referral of the matter to the central University EthicsCommittee or a requirement that the research be terminated.

Please retain this letter as formal recognition of the approval.

Yours sincerely,

Professor RS Phillips
Chair, FBLS Ethics Committee

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Dr Yannis Pitsiladis
Reader in Exercise Physiology and Director of ICEARS
IBLS Division of Integrated BiologyUniversity of Glasgow
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1/10/2008

Dear Dr Pitsiladis

FBLS Ethics Committee for Non Clinical Research Involving Human Subjects:
FBLS 0730 - The effects of hyperhydration on running economy of endurance trained runners

With regard to the application for an extension which you recently submitted to the FBLS Ethics Committee for consideration, I am pleased to let you know that the Committee has given its approval without qualification, for the period 1st June 2008 to 31st May 2009. Please advise the Committee if this time scale is exceeded. Please provide the committee with a short report on the project, including details of how the results of the project will be disseminated, by 30th June 2009. The committee is very interested to read the short report on completion of each project. Failure to submit the report by a researcher by the due date without reasonable excuse might result in a future application being refused. As a condition of approval and in line with the committee's need to monitor research, the following requirement applies: any unforeseen events which might affect the ethical conduct of the research, or which might provide grounds for discontinuing the study, must be reported immediately in writing to the ethics committee from which you have received approval. The committee will examine the circumstances and advise you of its decision, which may include referral of the matter to the central University Ethics Committee or a requirement that the research be terminated.

Please retain this letter as formal recognition of the approval.

Yours sincerely,

Professor RS Phillips

Chair, FBLS Ethics Committee

Professor RS Phillips

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