



**EFFECT OF TRANSPORT AND DELAYED ANALYSIS ON HEMATOLOGICAL VARIABLES
IN BLOOD SAMPLES TAKEN FROM ELITE SPEED SKATERS**

**UČINAK TRANSPORTA I ZAKAŠNJELE ANALIZE HEMATOLOŠKIH VARIJABLA
U UZORCIMA KRVI UZETIH OD VRHUNSKIH BRZIH KLIZAČA**

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SUMMARY

To study the effect of transport and storage on the stability of hematological variables, two blood samples from 81 elite short track speed skaters were analyzed within four hours after sampling. One sample was analyzed again 24 hours later. After analysis, the second sample was subjected to road and air transport and analyzed a third time 24 hours after the second analysis. To study the effect of storage up to 48 hours, one blood sample was taken from each of 81 elite long track speed skaters and subsequently analyzed within four hours after sampling, and again after 24 and 48 hours. Transporting the blood samples and storing them for 24 hours did not significantly change hemoglobin concentration and % reticulocytes. Hematocrit and the difference between total measured hemoglobin and cellular hemoglobin concentration were increased after 24 hours ($p < 0.01$). In samples stored for 48 hours, hemoglobin concentration remained stable for up to the entire 48-hour time period. In contrast, after 24 hours the mean cellular volume (MCV) increased ($p < 0.05$). After 48 hours hematocrit ($p < 0.05$), percentage and number of reticulocytes increased 48 hours after sampling ($p < 0.01$). In conclusion, hemoglobin concentration and percentage of reticulocytes may remain stable for up to 24 hours after sampling, while hematocrit may increase during this time period. Furthermore, hematocrit, MCV, and percentage and number of reticulocytes may also rise during the 24 hours after sampling. However, when transport is involved hemolysis may occur.

Keywords: sport, blood testing, storage, transport, hematology

SAŽETAK

Cilj rada bio je ispitati utjecaj transporta i skladištenja uzoraka krvi na stabilnost hematoloških varijabli.

Dva uzorka krvi uzeta od 81 vrhunska brza klizača na kratke staze analizirana su unutar 4 sata nakon uzimanja. Jedan je uzorak ponovno analiziran nakon 24 sata. Po prvoj analizi, drugi je uzorak transportiran, cestovnim i zračnim prometom te ponovno analiziran nakon 24 sata. Kako bi se odredio utjecaj skladištenja uzoraka tijekom 48 sati, u 31 vrhunska brza klizača na duge staze, jedan je uzorak krvi analiziran unutar 4 sata nakon uzimanja te ponovno nakon 24 i 48 sati.

Transport i skladištenje uzoraka krvi tijekom 24 sata nisu značajno promijenili koncentraciju hemoglobina i % retikulocita. Hematokrit te razlika između ukupnog hemoglobina i koncentracije staničnog hemoglobina povećala se nakon 24 sata ($p < 0.01$).

U uzorcima koji su bili uskladišteni tijekom 48 sata, koncentracija hemoglobina ostala je stabilna tijekom cjelokupnog vremenskog perioda. 24 sata nakon skladištenja došlo je do porasta srednjeg volumena eritrocita (MCV-a) ($p < 0.05$), a 48 sati nakon skladištenja došlo je i do porasta hematokrita ($p < 0.05$) te postotka i broja retikulocita ($p < 0.01$).

Koncentracija hemoglobina i postotak retikulocita ostaju stabilni i od 24 sata nakon uzimanja uzoraka krvi, dok hematokrit može porasti. Nakon 24 sata od uzimanja uzorka krvi raste hematokrit, MCV, postotak i broj retikulocita. Kada je uključen i transport uzoraka, može doći do njihove hemolize.

Ključne riječi: sport, testiranje krvi, skladištenje, transport, hematologija

INTRODUCTION

It has been demonstrated that an increase in hemoglobin mass may enhance maximal oxygen uptake and endurance performance (1, 2). Both blood boosting by infusion of autologous blood as well as administration of recombinant human erythropoietin (rhEPO) may be used in sports for performance enhancement, although these types of manipulation are forbidden (3). Since rh-EPO is only detectable in the urine within approximately 5 days after the last injection (4), some international sport federations attempt to deter blood manipulation by looking at indirect evidence for manipulation (5, 6, 7, 8). For this purpose hematological variables are measured such as hematocrit, hemoglobin concentration as well as markers of changes in erythropoiesis, i.e. number and percentage of reticulocytes (9, 10, 11, 12). In order to deter and detect manipulation with blood, the International Skating Union (ISU) started its blood testing program in 2000. For this purpose one day prior to selected events, a 3 ml blood sample is collected from all athletes (7). The two main variables that are used by the International Skating Union (ISU) to decide about start or no start are hemoglobin concentration and percentage reticulocytes, from which the so called OFF score can be calculated, being hemoglobin concentration in g/l minus 60 time the square root of % reticulocytes (13).

To ensure quality control the ISU has chosen to have the blood samples analyzed on an Advia 120 (Bayer Corporation, Tarrytown, NY, USA) calibrated with the sport protocol (14). At times this is available on site but in other circumstances the samples must be transported to a hospital and in those circumstances the samples would be analyzed 1-6 hours following sampling and transportation. As a consequence of this choice the samples may have to be transported, and between sampling and analysis 1-6 hours may elapse. When considering to increase the number of blood testing sites this may involve longer time between sampling and analysis. This does raise the question as to the hematological stability and analytical accuracy of samples that may have been transported and/or stored for several hours before analysis, in particular whether storage with or without transportation may yield a different hematological picture that may forbid an athlete to compete.

Therefore, in the present study we investigated the effect of storage up to 48 hours after sampling, with and without transportation on the main hematological variables that are used for blood testing in speed skaters (6). The blood samples were obtained from elite long track and short track speed skaters during regular blood testing.

MATERIAL AND METHODS

Subjects and blood collection

Because the blood samples were obtained during regular ISU blood testing (7), no approval from the Ethics Committee was required.

For studying the effect of transportation and storage for 24 hours, in 81 elite male and female short track speed

skaters participating in a World Cup event in Chicoutimi, Canada, February 2003, two K3 EDTA glass tubes of 3 ml each were filled with blood taken from a forearm vein using a vacutainer system (Sherwood medical, UK). Before venipuncture the athletes sat on a chair for at least five minutes, since it has been shown that after 5 minutes the hematological variables remain stable (15). Before blood sampling a tourniquet with moderate pressure was applied. As soon as the blood started to run, the tourniquet was released. The blood sampling was part of the ISU blood testing program (7).

For studying the effect of storage up to 48 hours, in 31 female elite long track speed skaters participating in a World Cup competition in Calgary, Canada, January 2003, one 3 ml blood sample was obtained from a forearm vein according to the same procedure as described above.

Handling of the samples

The two tubes from each short track speed skater were transported to the Chicoutimi hospital (5 minutes car drive) and analyzed between 2 and 4 hours after blood collection. Subsequently one tube from each athlete was placed in a refrigerator and kept at 4 ± 0.3 °C, while the second tube was transported by courier (Fed Ex) to Montreal, Canada, to the WADA accredited doping laboratory in a Styrofoam box with freezer packs that kept the temperature at approximately 4 °C. Transportation by road and air took approximately 5 hours in total.

Both samples from each athlete obtained in Chicoutimi, Canada, were analyzed again 24 hours after sampling, one in the Chicoutimi laboratory and the second one in the WADA (World Anti Doping Agency) accredited laboratory in Montreal.

The samples obtained in Calgary, Canada, in the long track speed skaters were analyzed approximately 1-3 hours after collection on an Advia-120 that was installed in a building adjacent to the ice rink, and after first analysis the tubes were stored in the refrigerator kept at 4 ± 0.3 °C, and re-analyzed 24 and 48 hours after collection.

Analysis and variables

The K3-EDTA tubes (Sherwood medical, UK) were placed on a blood mixer for 5 minutes prior to analysis by an Advia 120 (Bayer Corporation, Tarrytown, NY, USA). For calibration of the Advia-120 hematological analyzer, the Sport Calibration procedure was utilized (14).

For the purpose of the present study the following variables were measured: total hemoglobin concentration, the difference between total hemoglobin concentration and cellular hemoglobin concentration (delta Hb), hematocrit (%), number and percentage reticulocytes, and mean cellular volume (MCV)

Data handling and statistical analysis

The data were stored in an excel file, and analyzed using a statistical package (SPSS). To compare samples at various time points a repeated measures ANOVA was employed. When using analysis of variance (ANOVA) a post-hoc test was done to locate differences. A difference was considered statistically significant at $p < 0.05$. Data are presented as means \pm standard deviation.

RESULTS

As can be seen from Table 1, in the samples that were not subjected to several hours of transportation and were again analyzed 24 hours after sampling, mean hemoglobin concentration, percentage and number of reticulocytes were not significantly different. However, in samples that were transported for 5 hours from Chicoutimi to Montreal,

24 hours after sampling a significant difference was found in delta hemoglobin, which increased significantly from 0.1 to 0.4 g/dl ($p < 0.01$). In addition, mean hematocrit, measured within 4 hours after sampling were significantly lower than when measured 24 hours after sampling, while MCV was significantly increased from 24 hours after sampling. Transportation by itself did not significantly affect hematocrit (Table 1).

Table 1. Mean values (\pm sd) of hemoglobin concentration (Hb), hematocrit (Ht, in %), the difference between total and cellular hemoglobin concentration (Delta Hb). Percentage and number of reticulocytes (%Retic, and Retic#, respectively, expressed as number $\times 10^9/l$), Mean Cellular Volume (MCV), in samples that were measured 4 and 24 hours after sampling in Chicoutimi, as well as in samples that were measured in Chicoutimi, and subsequently transported to Montreal and measured again 24 hours after sampling. $n = 81$; * = $p < 0.05$; ** = $p < 0.01$, compared to the values 4 hours after sampling.

Tablica 1. Srednje vrijednosti (\pm sd) koncentracije hemoglobina (Hb), hematokrita (Ht, u %), razlika između ukupnog hemoglobina i stanične koncentracije hemoglobina (Delta Hb). Postotak i broj retikulocita (%Retic, i Retic#, odnosno prikazani kao broj $\times 10^9/l$). Srednji volumen eritrocita (MCV) u uzorcima koji su izmjereni 4 i 24 sata nakon uzimanja uzorka u Chicoutimi-u, kao i uzoraka koju su mjereni u Chicoutimi-u te potom transportirani u Montreal gdje su ponovno mjereni nakon 24 sata. $n = 81$; * = $p < 0.05$; ** = $p < 0.01$, u usporedbi s vrijednostima 4 sata nakon uzimanja uzoraka.

	Chic 4	Mon 4	Chic 24	Mont 24
Hb (g/dl)	14.7 \pm 1.1	14.7 \pm 1.1	14.7 \pm 1.2	14.7 \pm 1.2
Ht (%)	43.2 \pm 3.1	43.2 \pm 3.1	43.9 \pm 3.0*	43.7 \pm 4.7*
Delta Hb	0.1 \pm 0.2	0.1 \pm 0.2	0.1 \pm 0.1	0.4 \pm 0.1*
% Retics	1.2 \pm 0.3	1.2 \pm 0.3	1.2 \pm 0.3	1.3 \pm 0.3
Retic #	58 \pm 15	58 \pm 15	58 \pm 15	60 \pm 15
MCV (fl)	88.8 \pm 3.0	88.8 \pm 3.0	90.0 \pm 3.0*	91.4 \pm 3.2**

In Table 2, the results are presented from the samples that were collected in Calgary and stored for 48 hours after sampling. During 24 hours after sampling the main hematological variables did not change significantly compared to 1-3 hours of sampling except for MCV. However, 48 hours after sampling hematocrit increased significantly ($p < 0.05$) and also the percentage of reticulocytes as well as the number of reticulocytes were significantly increased ($p < 0.01$).

DISCUSSION

In the present study we investigated whether storage of blood samples with or without transportation may affect the variables that are used for the blood testing program used by the International Skating Union (ISU), up to 48 hours after sampling.

An important and consistent finding of the present study is that storage of blood samples up to 24 hours at 4 °C, did not significantly change mean hemoglobin concentration and percentage reticulocytes, when no transportation was involved. This finding is in line with two earlier studies. Kuipers et al (15) showed that the hematological variables as used by the ISU remained

stable for at least 8 hours after sampling and Parisotto et al (10) observed that the hematological variables would remain stable until 4 days after sampling when kept at 4°C. However, in contrast to the study from Parisotto et al (11) in the present study hematocrit was significantly increased 24 hours after sampling, whereas the percentage and number of reticulocytes tended to increase 24 hours after sampling, but were significantly increased 48 hours after sampling. This latter finding is somewhat surprising, since reticulocytes are supposed to ripen during storage and would expect to decrease in number and percentage. It is likely that the increase in reticulocyte number and percentage during storage is an artifact caused by cellular changes and staining properties over time, resulting in possible false positive identification of reticulocytes by automatic analyzers. This finding has to be taken into account, because percentage reticulocytes is used for calculation of the OFF score (13), which decides whether an athlete is allowed to start and whether or not follow up blood testing and/or urine testing for erythropoietin will take place (7). Although the increase in percentage reticulocytes 48 hours after sampling is likely to be an artifact, it does imply a limitation of elapsed time between sampling and analysis, because an increase in percentage

Table 2. Mean values (\pm sd) of hemoglobin concentration (Hb), hematocrit (Ht, in %), the difference between total and cellular hemoglobin concentration (Delta Hb). Percentage and number of reticulocytes (%Retic, and Retic#, respectively, expressed as number $\times 10^9/l$), Mean Cellular volume (MCV), in samples that were measured 1-3 hours after sampling, and again 24 and 48 hours after sampling in Calgary.

n = 31; * = $p < 0.05$; ** = $p < 0.01$, compared to values obtained within 4 hours after sampling.

Tablica 2. Srednje vrijednosti (\pm sd) koncentracije hemoglobina (Hb), hematokrita (Ht, u %), razlike između ukupnog hemoglobina i stanične koncentracije hemoglobina (Delta Hb). Postotak i broj retikulocita (%Retic, i Retic#, odnosno prikazani kao broj $\times 10^9/l$). Srednji volumen eritrocita (MCV) u uzorcima koji su izmjereni 1-3 sata nakon uzimanja uzoraka te ponovno nakon 24 i 48 sati nakon uzimanja uzoraka u Calgary-u.

n = 31; * = $p < 0.05$; ** = $p < 0.01$, u usporedbi s uzorcima uzetih unutar 4 sata nakon ispitivanja. that use percentage

	1-3 hrs	24 hrs	48 hrs
Hb (g/dl)	14.0 \pm 0.6	13.9 \pm 0.6	14.0 \pm 0.6
Ht (%)	42.6 \pm 1.6	42.7 \pm 1.6	43.3 \pm 1.6*
Delta Hb	-0.1 \pm 0.2	0.1 \pm 0.2	0.2 \pm 0.1
% Retics	1.3 \pm 0.3	1.3 \pm 0.3	1.7 \pm 0.4**
Retic #	62.0 \pm 16.9	63.5 \pm 15.6	80.4 \pm 19.0**
MCV (fl)	89.1 \pm 3.3	90.6 \pm 2.9*	91.4 \pm 2.8**

reticulocytes will cause changes in calculated variables that use percentage reticulocytes such as the OFF score (13). Another significant finding is that in samples measured from 24 hours after sampling, hematocrit may significantly increase, compared to the values found 4 hours after sampling. This is caused by cellular swelling as supported by an increase in MCV. The swelling of the red cell mass may be caused by depletion of glucose that is consumed by the red cells. Energy depletion of erythrocytes and swelling may lead to an increase in total red cell volume, and consequently in an increased hematocrit.

For sport practice an important finding of the present study is that samples obtained from athletes for blood testing can reliably be analyzed up to 24 hours after sampling for hemoglobin concentration and percentage reticulocytes. However, there may be some increase in hematocrit from 24 hours after sampling. Another relevant finding is that samples that are subjected to transportation and analyzed 24 hours after sampling may show an increase in free hemoglobin. The present study showed that difference between total and cellular hemoglobin concentration before transport was 0.1 g/dl and did increase after transport to 0.4 g/dl ($p < 0.01$). This may be due to hemolysis. However, the delta hemoglobin being the difference between cellular and total hemoglobin concentration as found in the samples that were subjected to both 24 storage and transportation was within the normal range. In addition, hematocrit increased slightly, which is incompatible with substantial hemolysis. This suggests that although some hemolysis may occur, the extent of hemolysis by transportation is limited.

Although the present study clearly demonstrates that blood samples can be reliably analyzed on an Advia 120 machine up to 24 hours after sampling for hemoglobin and percentage reticulocytes, it is unknown in how far this also holds for other hematological analyzers. As shown in the present study the increase in percentage reticulocytes 48 hours after sampling is probably an artifact. Robinson et al (16) have shown that systematic differences in reticulocyte count may exist between different modern hematological analyzers, and it is unknown in how far reticulocyte count and reticulocyte parameters may be affected by storage.

In summary, the present study shows that hemoglobin concentration and percentage reticulocytes do not significantly change up to 24 hours after sampling, when the samples are kept refrigerated at 4 °C and not subject to transportation. Transportation may induce hemolysis, thereby increasing the difference between total and cellular hemoglobin concentration. With storage beyond 24 hours the percentage and number of reticulocytes as well as hematocrit does increase. Therefore it is recommended to analyze blood samples in athletes within 24 hours.

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References

1. Birkeland KI, Stray-Gundersen J, Hemmersbach P, et al. Effect of rhEPO administration on serum levels of sTfR and cycling performance. *Med Sci Sports Exerc* 2000; 32: 1238-43
2. Gore CJ, Hahn G, Burge CM, et al. VO₂max and hemoglobin mass of trained athletes during high intensity training. *Int J Sports Med* 1997; 18: 477-82.
3. WADA., 2005: www.wada-ama.org/en
4. Lasne F, Ceaurriz Jde. Recombinant erythropoietin in urine. *Nature* 2000; 405: 63.
5. FIS rules 2005: www.fis-ski.com/rulesandpublications/medicaldoping.html
6. IBU, 2005. www.biathlonworld.com/media/media_0007920.PDF
7. ISU. Communication 1352, (www.isu.org), 2005
8. UCI, 2003: www.uci.ch/english/about/rules_2004?ch13.pdf
9. Lippi G, Guidi G. Laboratory screening for erythropoietin abuse in sport: an emerging challenge. *Clin Chem Lab Med* 2000; 38: 13-9.
10. Parisotto R, Gore CJ, Hahn AG, et al. Reticulocyte parameters as potential discriminators of recombinant human erythropoietin abuse in elite athletes. *Int J Sports Med* 2000; 21: 471-9.
11. Parisotto R, Wu M, Ashenden MJ, et al. Detection of recombinant human erythropoietin abuse in athletes utilizing markers of altered erythropoiesis. *Haematologica* 2001; 86: 128-37.
12. Schmidt W, Bierman B, Winchenbach P, et al. How valid is the determination of hematocrit values to detect blood manipulations? *Int J Sports Med* 2000; 21: 133-8.
13. Gore CJ, Parisotto R, Ashenden MJ, et al. Second generation blood tests to detect erythropoietin abuse in athletes. *Haematologica* 2003; 88: 333-44.
14. Kling G. Using the ADVIA 120 for Sports Events: Calibration Procedures, Internal Report Bayer Corporation, 2000.
15. Kuipers H, Brouwer T, Dubravčić-Simunjak S, et al. Hemoglobin and hematocrit values after saline infusion and tourniquet. *Int J Sports Med* 2005; 26: 405-8.
16. Robinson N, Schattenberg L, Zorzoli M, et al. Haematological analysis conducted at the departure of the Tour de France 2001. *Int J Sports Med* 2005; 26: 200-7.