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The Effects of Swimming on Blood Nitric Oxide and Haematological Parameters

Yüzmenin Kan Nitrik Oksit ve Hematolojik Parametreler Üzerine Etkisi

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ABSTRACT

Background: Nitric oxide (NO) is a gas with atherosclerosis-inhibiting effect. NO is also involved in the structure and function of erythrocytes (RBCs). Blood NO levels and haematological parameters (HPs) and the relationship between these parameters in swimmers over one-year training season have not yet been evaluated.

Material and Methods: The effects of training (TP), detraining (DTP), and retraining period (RTP) on blood NOx (as total nitrite) levels and the relationships between NO and HPs in child swimmers were investigated. Ten trained male swimmers (11.1±0.6 years old) joined the study. With intervals of two months; blood biochemistry, physical and physiological measurements were performed after TP, DTP, and RTP. As an endurance criterion, critical speed (CS) was measured. Venous blood was obtained after 12 hours of fasting, serum iron, ferritin, total iron binding capacity, NOx (by Cd^{++} mediated 'Griess' assay) levels were measured, and haemograms were assessed.

Results: NOx levels during TP were below the baseline levels (19.3%) and increased to the baseline during DTP. NOx was greater in RTP compared with DTP ($p<0.05$) and TP ($p<0.01$). Oxidative stress index (total oxidant status/total antioxidant status ratio) at TP was greater than DTP and RTP ($p<0.01$). WBC, RBC, Hgb and Hct were lower in DTP compared with TP ($p<0.01$). TP's iron (below normal ranges) and ferritin (at the borderline) levels were increased in DTP. NOx levels were significantly correlated with ALT ($r=-0.68$, $p<0.05$) and MCV ($r=-0.73$, $p<0.05$) in TP, and with RBC ($r=-0.66$, $p<0.05$) in DTP. No significant relationship was found between CS and NOx or HPs.

Conclusions: After eight-months of TP, NOx levels were below the baseline and created therisk of anemia. These negative effects were recovered at DTP and improved at RTP. The decreased levels of NOx in TP may be due to the role ofhigh oxidative stress, as well as high consumption of NOx by increased RBC and Hb levels that occurred by a possible hypoxia caused by a long-term training.

Keywords: Nitric oxide, red blood cell, haemoglobin, swimming, training, retraining

ÖZ

Giriş: Nitrik oksit (NO), aterosklerozu engelleyici etkilere sahip bir gazdır. NO, aynı zamanda eritrositlerin (KKH) yapısı ve fonksiyonunda da rol alır. Bir yıllık yüzme sezonunun çocuklarda kan NO düzeyleri ve hematolojik parametreler (HPs) ile buparametreler arasındaki ilişkiler üzerine etkileri bilinmemektedir.

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Amaç: Bu çalışmada sekiz aylık bir antrenman periyodu (AP), deantrenman periyodu (DAP) ve reantrenman (RAP) periyotlarının kan NOx (total nitrit olarak) ve HPs'ler üzerine etkileri ve bu parametreler arasındaki ilişkiler araştırıldı.

Gereç ve Yöntemler: Çalışmaya yaş ortalaması (11.1±0.6 yıl) olan 10 sağlıklı antrene yüzücü erkek çocuk katıldı. İki ay aralıklarla; AP, DAP ve RAP sonrası kan biyokimyası, fiziksel ve fizyolojik ölçümler yapıldı. Dayanıklılık kriteri olarak; kritik hız (KH) ölçüldü. Oniki saatlik açlık sonrası alınan venöz kan örneklerinden hemogram; serumda demir, ferritin, total demir bağlama kapasitesi ve NOx (Cd^{++} aracılı 'Griess' yöntemi ile) düzeyleri ölçüldü.

Bulgular: NOx değeri, AP'de bazal değerlerinin altında (19.3%) iken DAP'de artarak bazal değerlerine döndü. RAP'de anlamlı olarak arttı ($p<0.05$). AP'deki lökosit (BKH), **KKH**, alanin aminotranferaz aktivitesi (ALT), hemoglobin (Hgb) ve hematokrit (Hct) değerleri, DAP'de azalırken ($p<0.01$), AP'de normal değerlerinin altında bulunan serumda demir ve anemi sınırındaki ferritin düzeyleri DAP'de normal değerlerine ulaştı. AP'de yüksek düzeyde oksidatif stres (total oksidan statüsü/total antioksidan statüsü oranı) tespit edildi. Kan NOx düzeyleri; AP'de; ALT ile ($r=-0.68$, $p<0.05$) ve DTP'de ortalama eritrosit hacmi ile ($r=-0.73$, $p<0.05$); DTP'de KKH ile ($r=-0.661$, $p<0.05$) ilişkili bulundu. KH ile diğer parametreler arasında anlamlı bir ilişki bulunmadı.

Sonuç: Sekiz aylık AP NOx düzeylerini düşürdü ve anemi riski yarattı. DAP bu negatif etkileri düzeltti. RAP, NOx düzeylerini iyileştirdi. AP'de NOx düzeylerinin düşük bulunmasında, bu dönemdeki yüksek oksidatif stres düzeyinin yanı sıra, muhtemel bir antrenman hipoksisi nedeniyle artan KKH ve Hgb'nin fazla miktarda NOx'u tüketmiş olmasının da rolü bulunabilir.

Anahtar Sözcükler: Nitrik oksit, kırmızı kan hücresi, hemoglobin, antrenman, deantrenman, reantrenman

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INTRODUCTION

Nitric oxide (NO) has vasodilator, antioxidant and some metabolic regulatory properties including regulation of function and integrity of erythrocytes (RBCs) (1). Synthesis of NO from L-arginine is catalyzed by nitric oxide synthase (NOS). NOS has three isoforms: Type-I (neuronal NOS, nNOS), Type-II (inducible NOS, iNOS) and Type-III (endothelin NOS, eNOS) (1). Besides being a potent vasodilator, NO reduces the invasion of leukocytes into the intimae, platelet aggregation, and vascular smooth muscle proliferation. NO is an independent risk factor of long-term cardiovascular morbidity and mortality (1-3). Therefore, it is important to know blood NO levels and to improve it through exercise.

NO has a central role for the regulation of vascular tone and cardiovascular signaling mechanisms. In these mechanisms, hemoglobin (Hgb) plays the role of an important allosteric protein. Mechanisms proposed for RBCs contain the ability of Hgb to sense changes in oxygen concentrations in tissues, and combining this function to regulating NO levels in circulation (4). Under normoxic conditions, nitrite is oxidized by oxyhemoglobin, producing nitrate and methemoglobin, and preventing NO vasodilatory effects. Vasoactive NO that provides vasodilatation is created when the partial pressure of oxygen decreases and nitrite is reduced by the deoxygenated Hgb (5). Scavenging NO by cell-free hemoglobin may also have an important role in pathological processes of hemolysis (6). In addition, it was indicated that RBCs consume major amounts of NO from the circulation when erythrocytes are hypoxic (7,8). Therefore, these factors can limit NO bioactivity. Accordingly, the sensitivity of eNOS, RBC and Hgb levels decreased significantly in hockey players after a training camp, due to hemolysis (9). Similarly, in trained rodents, high intensity exercise impaired the characteristics of RBCs' plasma membrane, resulting in diminished deformability (10). Although it was exhibited that three months of intensive swim exercise induced a significant increase in NOx (nitrate and nitrite), it decreased iron levels both in plasma and tissues in rats (11). These findings can exhibit that long-term swimming season may initialize some health

risks as anemia, and decreases in NOx levels in children (1,6).

Swimming training with high intensity and volume is applied in most countries, including Turkey, where it is well known. However, the effects of annual swimming training of hypoxic nature on NO and RBC metabolism in children are not known. In addition, the relationships between NO and RBC provide significant knowledge about the effects of training that has been applied in an optimum training planning. Thus, we hypothesized that long-term swimming training (TP) and retraining (RTP) periods can decrease blood NOx levels and produce anemia, and that detraining period (DTP) recovers these harmful effects. We investigated the relations between NOx levels and haematological parameters (HPs) in young male swimmers.

MATERIAL and METHODS

Study Groups

The study group consisted of 10 healthy, trained male swimmers (11.1±0.6 years old), who were active members of the Ege University swimming club. Three measurements were planned. The initial measurement session (TP) was performed four days after the Turkish National Swimming Championship (consiting of two days of four sessions), which was held at the end of the swimming season. The second measurement took place after two months of detraining period (DTP, in off-season), and the last measurement was performed eight weeks after retraining period (RTP, at the beginning of next season), subsequently. TP was the eight months' of continued training period, until DTP.

The aim, benefits, test applications, possible risks were explained to participating children and parents, and signed informed consents were obtained from the parents. Ege University Medical Faculty's Ethics Committee approved the study.

Excluding Criteria and Warnings

Swimmers were warned not to modify their diets the week prior to the tests and not to train hard the last three days. In addition, participants were warned not to carry out regular exercise or physical activity, and not to take any medicine, vitamin supply or similar matters which would effect the oxidative defense system. Failure to obey the warnings mentioned and occurrence of any sickness were accepted as excluding criteria.

Training Details

At TP, training frequency was six days per week; total volume was between 25 and 30 km, training intensity was ranged between the 2.0 and 4.0 mM lactate thresholds. At RTP, swimmers trained one session a day and six days a week. In the first week, they swam 18 km in total; in the second week total volume was increased to 21 km; and in the third week it reached a volume of 24 to 27 km. Continuous and interval loading methods were used in the training sessions. Exercise intensities ranged between the 2.0 mM and 4.0 mM lactate thresholds. At DTP children were asked to be away from physical activity, except swimming with low frequency and volume.

Procedures

Body measurements (height, body weight) and body composition were measured using a Tanita SC-330 S body composition analyzer (Tanita, Tokyo, Japan).

Body Mass Index (BMI)

BMI was calculated via height and body weight values according to following formula: BMI= weight (kg) / height $(m)^2$.

Swimming Performance Test (Critical Speed)

Critical speed is used as a criterion of aerobic endurance capacity, which is calculated according to following formula via maximum 50m and 400m freestyle swimming times in seconds, performed with one day break, and full resting; Critical speed (in m/s) = (400m-50m) / (400m time (s) - 50m time (s)), according to reference (12). Whole measurements including physical

and exercise tests were realized in the morning between 09:00-11:00, subsequent to 12 hours of fasting. Subjects were warned about not to do any change in their usual nutrition diets, and not to do intensive exercises at least three days prior to the tests.

Obtaining and Analysis of Blood Samples

Subjects' overnight venous blood samples were taken into cooled vacuumed 3.0 ml tubes with EDTA for haemograms, and into 8.0 ml serum tubes. Haemogram analysis was performed by auto analyzer (BC-3000 Plus, Mindray, PRC). Haemograms included RBC, leukocyte (WBC), haematocrit (Hct), haemoglobin (Hgb), mean erythrocyte volume (MCV) and thrombocyte (Plt) parameters. Serum tubes were centrifuged at 2000g for 10 min, and remained for 30 min at room temperature. Serum samples were kept at -70°C, until the biochemical assays were performed in a single batch. Biochemical parameters were measured from serum samples within a month.

Analysis of Blood NOx Levels

Nitrite is the main product of NO oxidation in plasma, and it was demonstrated that the concentration of nitrite accurately reflects changes in NOS activity (13). Therefore, blood NOx (as total nitrite), nitrite in the sample and nitrite reduced from nitrate using cadmium (Cd^{++}) was assayed at 540 nm by using a nonenzymatic colorimetric kit (NB-88; Oxford Biomedical Research, Oxford, MI, USA), with the Griess reagent, as described by the manufacturer, through a microplate reader (DialabGmbh, Diareader ELx800G, Austria). The coefficients of variation (CV) for intra- and inter-assay of the NO kit were 7.9% and <10%, respectively.

Other Biochemical Parameters

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) enzyme activities, serum iron, ferritin and iron binding capacity (TIBC) levels were also analyzed via standard kits (DialabGmbh, Austria) by auto analyzer (Beckman, CX7, Brea, Ca, USA).

Statistical Analyses

Data were analyzed using SPSS for Windows (Release 22 Chicago, IL, USA). The Kolmogorov-Smirnov test was applied to determine whether data were normally distributed, and it revealed normal distribution. Therefore, parametric analysis methods were used. Means and standart deviations (SDs) were used for description. Binary comparisons were done by paired t-test. The Pearson test was used for correlation analysis. G*Power 3.1 analysis used the 0.05 level for significance. Statistical power analysis program (14) was used to test the effect size (d) and power (pw, as 1-β approaches 1.0) of the statistical test used for differences in NOx and TAS.

RESULTS

Physical and Physiological Data

Body weight increased significantly between TP and DTP, and then decreased after RTP, while body mass index (BMI) was only decreased at RTP. Height and critical speed (CS) increased significantly throughout the periods (Table 1).

Haematological Parameters (HPs)

WBC, Hgb, Hct and TIBC (normal range 250-400 µg/dl) levels were significantly higher in TP while serum iron (normal range 50-120 µg/dl) and ferritin (normal range: 20-300 ng/ml) were lower in TP than those in DTP, and the serum iron/TIBC ratio was 12% in TP, when compared with DTP. RBC decreased insignificantly from TP to DTP, but increased significantly between DTP and RTP. MCV (normal range 76-90 fl, except in TP) and TIBC decreased significantly between DTP and RTP, but PLT remained unchanged following any period (Table 2). Hematological parameters were in their normal ranges (except for iron at TP, MCV at DTP and RTP, and TIBC at RTP) during periods.

Biochemical Parameters

Blood NOx levels increased insignificantly between TP and DTP (19.3%), but the statistical power (pw=0.522) and effect size (d=0.714) of this increase was strong enough. NOx levels increased again between DTP and RTP ($p<0.05$). NOx level in RTP was higher than that in TP ($p<0.01$) (Table 2 and Figure 1). ALT levels decreased significantly from TP to DTP ($p<0.01$). There was no significant difference among periods for AST activities (Table 3).

Table 1. Physical and physiological measurement data and comparisons

	TP	**t** (TP/DTP)	**DTP**	**t** (DTP/RTP)	**RTP**	**t** (RTP/TP)
Weight (kg)	47.6 ± 8.2	-9.31**	50.9 ± 8.8	2.05	50.3 ± 8.6	10.3**
Height (m)	1.52 ± 0.96	-12.3**	1.56 ± 0.10	-6.13**	1.58 ± 0.10	13.6**
BMI (kg/m^2)	20.4 ± 2.6	-2.17	20.8 ± 2.7	4.25**	20.0 ± 2.5	2.32
CS (m/s)	1.03 ± 0.07	-3.21*	1.04 ±0.07	-5.46**	1.07 ± 0.08	6.53**

Data as mean ± SD; BMI: body mass index, CS: critical speed. Comparison of periods, *: $p<0.05$, **: $p<0.01$

Table 2. Hematological measurement data and comparisons

	TP	**t** (TP/DTP)	**DTP**	**t** (DTP/RTP)	**RTP**	**t** (RTP/TP)
WBC ($10^3/\mu l$)	7.42 ± 1.74	3.35**	5.37 ± 1.73	0.78	5.12 ± 1.25	5.12**
RBC ($10^6/\mu l$)	5.17 ± 0.38	0.68	5.08 ± 0.35	-4.09**	5.50 ± 0.40	-2.43*
Hgb (g/dl)	13.8 ± 1.0	5.62**	12.7 ± 0.8	0.17	12.7 ± 1.0	4.96**
Hct (%)	40.8 ± 2.7	5.99**	37.3 ± 2.2	-1.73	38.5 ± 2.3	2.65*
MCV (fl)	79.0 ± 4.2	4.04**	73.7 ± 5.3	10.9**	70.3 ± 5.1	6.67**
Ferritin (ng/ml)	24.4 ± 8.8	-1.88	31.8 ± 16.1	1.25	28.3 ± 12.4	-1.55*
PLT ($10^3/\mu l$)	305 ± 70	-0.47	311 ± 66	1.04	312 ± 68	1.33
Iron (g/dl)	38.3 ± 9.1	-7.86**	71.6 ± 13.3	-1.22	79.5 ± 30.0	-4.51**
TIBC (µg/dl)	317 ± 66	3.05*	264 ± 75	3.52*	213 ± 92	3.76**

Data as mean ± SD; WBC: white blood cells, RBC: red blood cells, Hgb: haemoglobin, Hct: haematocrit, MCV: mean cell volume, PLT: platelet, TIBC: total iron binding capacity. Comparison of periods, *: $p<0.05$, **: $p<0.01$

Table 3. Biochemical parameter measurement data and comparisons

	TP	**t** (TP/DTP)	**DTP**	**t** (DTP/RTP)	**RTP**	**t** (RTP/TP)
NOx (µmol/l)	44.4 ± 17.1	-1.11	52.9 ± 27.8	-2.58*	68.0 ± 16.4	-3.98**
AST (U/l)	23.3 ± 3.0	0.75	22.8 ± 2.1	-1.30	23.5 ± 4.4	0.31
ALT (U/l)	22.9 ± 4.2	5.38**	16.1 ± 3.9	0.41	15.7 ± 5.0	4.28**

Data as mean ± SD; NOx: nitric oxide (as total nitrite), AST: aspartate aminotransferase, ALT: alanine aminotransferase. Comparison of periods, *: $p<0.05$, **: $p<0.01$

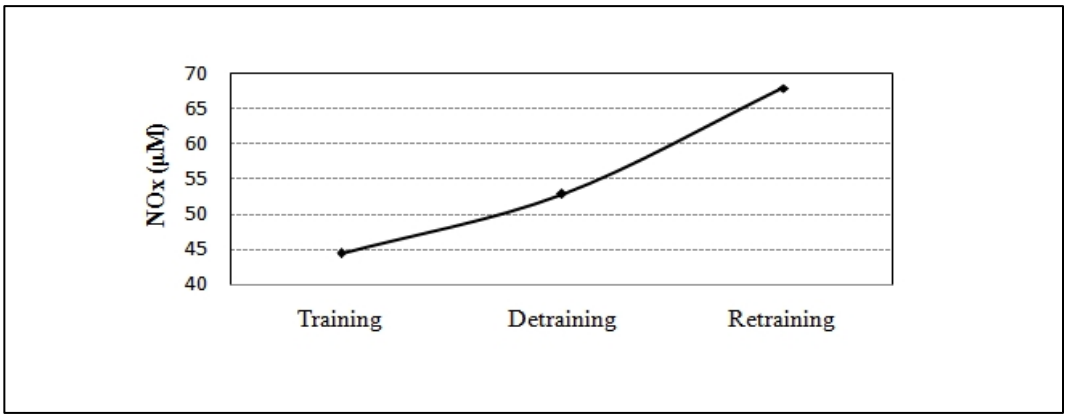

Figure 1. The changes of nitric oxide (NOx, as total nitrate) during training, detraining and retraining

Main correlations: NOx was significantly correlated with ALT (r=-0.68, p<0.05) and MCV (r=-0.73, p<0.05) in TP, while NOx showed a milder relation with RBC (r=-0.66, p<0.05) and BMI (r=-0.67, p<0.05) in DTP.

DISCUSSION

The main findings of the present study were that TP decreased below baseline NOx levels and created "iron deficiency without anemia" (IDWA), contrary to RTP. DTP corrected these harmful effects. Decreased NOx in TP was related to increased MCV levels. These results partially confirm our hypotheses.

The Effects of TP, DTP and RTP on NOx Levels

It was exhibited that, after about two-months of detraining, NOx levels in the blood returned to baseline values (15,16). Thus, increased NOx values at DTP according to TP can be accepted as basal values. These results indicate that NOx levels falled below basal levels during TP or the end of TP contrary to RTP. Both acute (13) and regular aerobic exercises in swimmers (17) and in sedentaries (15) improved blood NOx levels similarly to RTP.

The reduced NOx levels in TP can be explained by depending on two reasons. The first main reason can be the extremely increased oxidative stress at the end of TP. The second reasonmay be the interactions between NOx and RBC due to hypoxia. When the initial reason is considered, blood oxidant stress index (OSI: TOS/TAS) and TOS levels were found to be greater in TP than DTP and RTP (p<0.01)(18). In our another published study carried out with the same swimmers and at the same periods with the present study(18), the difference was also moderately strong (d=0.77, pw=0.592). These results reveal the presence of an extreme oxidative stress in TP where TAS is depressed. This extreme oxidative stress in TP might have been caused due to the national competitions which was held at the end of TP, rather than the training itself.

It is known that intensive aerobic exercise such as in swimming can increase oxidative stress (1,19), and that oxidative stress also inhibits NOS activity and decreases blood NO levels (1,2). Other reactive oxygen species' (ROS) sources have relatively low oxygen demand (20) and are inflammatory agents that occur during exercise (21). Furthermore, it is reported that children may be more susceptible to extreme oxidative stress by chronic exercise (22). Oxidative stress is related to inflammation (23). In the present study, ALT and WBC levels as markers of muscle injury (24) and delayed-onset of muscle soreness (DOMS) (25), were higher in TP than DTP and RTP. Therefore, despite being in their normal range, these increased inflammation markers in TP may be a result of intensive national competitions performed at the end of TP.

In addition, a negative relationship was found between NOx and ALT at TP. Therefore, these increased imflammation markers and this

relationship at TP confirms our view that the increased oxidative stress at TP can decrease NOx levels. The best established stimulus on NO production is the shear stress produced in the blood stream, which can increase NOS enzyme activity (1,26). Exercise training stimulates endothelial cells' NO synthesis (1). At the cellular level, shear stress produced by injured fibers within muscle is thought to induce synthesis of NO by neuronal NOS in beneath the sarcolemma and eNOS in muscle smooth (26). RBC-NOS activity is also stimulated by shear stress and mechanical deformation of RBC (27) .Therefore, shear stress can also have a role in the increase of NOx in RTP under moderate-oxidative stress conditions ($p<0.05$) according to TP.

Furthermore, swimming training also caused increased antioxidants in children from 10 to 13 years of age (28). Therefore, the swimmers have an antioxidant capacity (increased TAS in RTP) enough to neutralize moderate oxidative stress, which may also play arole in the increase of NOx at RTP. Besides, all isoforms of NOS can be regulated by transcription with hypoxia (1). Therefore, the hypoxic nature of swimming in the heat, the hydrostatic pressure and physical or physiological movement forms can also affect an increase in NO levels in both RTP and TP (1).

RBC and the Relations with NOx

Serum iron levels were found to be decreased to the lowest level and under the normal range, and ferritine was found to be at the lowest levels and the iron/TIBC ratio was 12%, as well as reduced NOx in TP comparing with DTP (Table 2). TIBC, Hct, Hgb and RBC were lower at DTP contrary to TP. However, NOx was greater than TP. If haemoglobin levels are normal against a depressed serum ferritin concentration, this status is defined as "iron deficiency without anemia" (IDWA) (29). The results can imply an IDWA situation in TP, but not also in other periods. IDWA might also be one of the causes leading to changes in iron metabolism in response to long-term exercise training by shifting iron from storage sites and increasing iron turnover (11,30), with accompanying nutritional deficiency.

It was reported that the increased NO induced by exercise might lead to increased transferrin-receptor number on the cellular membrane and transferrin-bound iron uptake by bone marrow cells, decreased plasma iron and iron storage by iron utilization. In addition, unexpected increase (return to baseline) of NOx levels at DTP may be a result of the decrease in the Hgb and RBC levels together with disappearance of possible hypoxia, as well as high oxidative stress at DTP. It was established that intensive short-term hypobaric hypoxia can cause local hypoxic situations, and activate erythropoietic response respectively (31). Therefore, increased RBC, Hct, MCV and Hgb at TP might have been caused by increased erythropoietin due to hypoxia and IDWA to tolerate the conditions.

It is also reported that hypoxic RBCs consume large volumes of NO (8). MCVs were at their highest levels at TP than at DTP. In addition to the aforementioned, a negative relationship was found between NOx and MCV at TP. This relationship shows that increasing MCV levels of RBC can decrease the amount of NOx in hypoxic conditions at TP. These results confirm our opinion that hypoxic conditions can reduce blood NOx levels in child swimmers.

Suhr et al. (9) found a significant decrease in Hgb and Hct levels after an intensive training camp in hockey players. Unlike in the previous study, Hgb and Hct at TP were greater than DTP, four days following the national competitions. Therefore, the reason of decrease in the last two parameters in DTP may not be RBC hemolysis as in the previous study. The negative relationship between NOx and RBC at DTP confirms the hypothesis that under normoxic conditions nitrite is oxidized by oxyhemoglobin producing nitrate (5), contrary to hypoxic TP. Therefore, the main reason of the increase in NOx in DTP can be explained by this hypothesis, as well as reduced oxidative stress.

The reason of the absence of any relationship between NOx and RBC at RTP, despite increased TOS, RBC, Hct and NOx levels in relation to DTP may be mainly the absence of hypoxia, and the intensity, frequency and volume of training due

to starting a new swimming season. Therefore, a well-balanced nutrition strategy and persistent observation of swimmers in whole periods are necessary in view of anemia risk.

The present study is the first human study inthe literature, which displays the role of NO in anemia risk at TP during long-term swimming training in children. However, in contrast to our expectations (1), no significant relationship between endurance capacity (CS) with NOx was found. Therefore, it can be said that the changes in blood NOx and HPs occurred independently from the CS.

Limitations

The number of participants in the study was a limitation. The major competition which was held at the end of TP may have affected the results at TP. Thus, we were unable to reveal the true impact of TP. However, this holding is still used in many countries developed in the swimming sport. However, this study gave us an opportunity to show the effects of hypoxia with oxidative stress on NOx and haematological parameters in details in humans, in the pool instead of laboratory conditions.

CONCLUSION

TP decreased NOx and created anemia risk at TP, whereas the DTP recovered them. RTP improved NOx levels. The reasons of the decrease in NOx at TP may be mainly attached to oxidative stress, as well as the high consumption of NOx by increased RBC and Hgb levels that have occurred by a possible hypoxia due to training.

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