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Association of endothelial nitric oxide synthase gene G894T polymorphism with blood oxygen transport

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ABSTRACT

Background: Nitric oxide (NO) is involved in formation of oxygen transport function of blood and oxygen transport in tissues by means of interaction with haemoglobin. Endothelial nitric oxide synthase gene G894T polymorphism affects the expression and the activity of the NO synthase enzyme. The influence of this polymorphism on the formation of oxygen-dependent processes in the body has not been completely investigated.
Objective: To evaluate the associations between G894T polymorphism of the endothelial nitric oxide synthase gene and the condition of oxygen transport function of blood in healthy male volunteers.
Methods: The study subjects were healthy young males aged 18–24 years (n = 165). The blood was drawn from the cubital vein at rest after 12 h following the last food intake. G984T polymorphism and the blood oxygen indices pO_2, CvO_2, SO_2, pH, $p50_{stand}$ (the temperature was 37°C, pH = 7.4, pCO_2 = 40 mm Hg) and $p50_{act}$ (actual temperature, pH and pCO_2), etc. were determined.
Results: In persons with the TT-genotype, the oxygen content in venous blood was 48.7% (q = 0.019) lower compared to subjects with the GT-genotype and 49.4% (q = 0.019) lower compared to subjects with the GG-genotype. The saturation of blood in carriers of the TT-genotype was 32.4% (q = 0.021) and 35.9% (q = 0.019) lower as opposed to subjects with the GT-genotype and the GG-genotype, respectively. In blood of subjects with the TT-genotype, the oxygen tension was 26.1% (q = 0.019) lower as compared to subjects with the GT-genotype and 27.4% (q = 0.019) lower as opposed to the GG-genotype. In turn, volunteers having a common allele in their genotype (GG + GT) demonstrated oxygen tension to be 26.7% (q = 0.019) higher compared to subjects with the TT-genotype. The blood pH values of the subjects having the recessive genotype were 0.022 units lower compared to the GG (q = 0.044) and GT (q = 0.042) genotypes. In volunteers with the TT-genotype, the $p50_{stand}$ parameter was 5.8% (q = 0.027) lower compared to subjects with the GT-genotype and 6.8% (q = 0.019) lower compared to volunteers with the GG-genotype. In persons with two T-alleles in their genotype, $p50_{act}$ was 5.4% (q = 0.019) lower compared to subjects with the GT-genotype and 6.4% (q = 0.019) lower compared to persons with the GG-genotype.
Conclusion: The T-allele of G894T polymorphism is associated with low values for oxygen content, oxygen tension, acidic pH shift and increased haemoglobin affinity for oxygen under standard and real conditions. The presence of a minor allele in G894T polymorphism of the NOS3 gene contributes to formation of oxygen transport function of blood.

1. Introduction

Nitric oxide (NO) is a simple compound that plays an important physiological role in the body, regulating vascular tone, as well as cardiovascular, respiratory, digestive, immune, nervous and other physiologic systems, and possessing free-radical and antioxidant properties [1]. Research into physiological effects of NO has many aspects, namely its interaction with different components of blood, particularly with haemoglobin. Corpuscular hemoglobin plays a significant role in NO homeostasis [2]. NO can exert its influence through different mechanisms: haemoglobin oxygen affinity modification, vascular tone regulation, peroxynitrite formation and effects [3]. In addition to the above effects, NO is involved in performing oxygen transport function of blood and oxygen transport in tissues by means of interaction with haemoglobin [4]. In particular, NO is a kind of an allosteric regulator of haemoglobin functional activity by means of formation of various NO forms: S-nitrosohaemoglobin, nitrosilhaemoglobin, and methaemoglobin, which play significant roles in oxygen transport function of

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blood via modification of haemoglobin affinity for oxygen [5].

Regulation of the expression of endothelial NO synthase (NOS3), which is responsible for formation of NO in the body, is encoded by a respective gene located in the 7q35-7q36 region of chromosome 7 [6]. A few polymorphisms of the gene are responsible for various manifestations of physical features and cause development of various pathologies. Of interest is G894T polymorphism (Glu298Asp, rs1799983), which is responsible for substitution of guanine by thymine in position 894 in exon 7, which leads to substitution of glutamate by aspartate in position 298 of the NOS3 gene [7]. The influence of this polymorphism on the formation of oxygen-dependent processes in the body has not been completely investigated. Therefore, the goal of this study was to evaluate the associations between G894T polymorphism of the endothelial nitric oxide synthase gene and the condition of oxygen transport function of blood in healthy male volunteers.

2. Methods

2.1. Subjects

The study subjects were healthy young non-smoking males aged 18–24 years (n = 165), residents of the Republic of Belarus. The level of their physical activity averaged to 150–300 min per week. The subjects confirmed their voluntary participation in the study by a written informed consent. The study was approved by the Biomedical Ethics Committee of Grodno State Medical University (Belarus). To determine polymorphism of the NOS3 gene and blood oxygen, blood was drawn from the cubital vein at rest 12 h after the last food intake.

2.2. Determination of G984T polymorphism

G984T polymorphism was determined by allele-specific polymerase chain reaction with real-time fluorescence detection. The blood was drawn from the cubital vein at rest 12 h after the last food intake. For blood sampling 3 ml EDTA K3 Vacuette Tube (VACUETTE®, Austria) was used. DNA was extracted using the XpressDNA™ Blood kit (Lytech Co. Ltd., Russia) that allows genomic DNA extraction from whole blood leucocytes. Whole blood (1000 mcl) collected in an Eppendorf safe-lock tube was centrifuged at 3000 rpm for 15 min at room temperature. After centrifugation, the blood was separated into plasma and formed elements. Then the plasma was removed and the locked tubes were kept at - 20°C for 1 h until complete freezing of the formed elements was achieved. After that the tube contents were thawed at room temperature. Further the DNA-XPress™ reagent was added and thoroughly mixed with the tube contents for 10 s using a rotary shaker (vortex). The tube was then placed into a thermostat heated to 98 °C for 15 min. Finally, the tube contents were centrifuged at 14,000 rpm for 15 s at room temperature. The obtained supernatant was used as a DNA sample for further studying.

G984T polymorphism was determined using a set of reagents (Syntol, Russia), which included Taq DNA polymerase, diluent, 2.5-fold reaction mixture with two allele-specific probes labeled with different fluorophores. Amplification of the DNA locus studied was performed using a Rotor Gene-Q system (Qiagen, Germany). Allele discrimination was performed with the amplifier software system (Q-Rex Software) that relies on the dependence of fluorescence intensity of the corresponding dye and the number of copies of the studied region of the gene.

2.3. Determination of blood oxygen

For blood gases evaluation a 2-ml Heparin Vacuum Tube (VACUETTE®, Austria) was used. The test was performed within 3 h after blood sampling. A Stat Profile pHOx Plus L (NOVA Biomedical, USA) gas analyser was used to determine oxygen tension (pO_2), carbon dioxide tension (pCO_2), oxygen content (CvO_2), blood oxygen saturation (SO_2), haemoglobin level (Hb), methaemoglobin level (MetHb), oxygen capacity of blood (OC), pH, bicarbonate concentration (HCO_3^-), standard bicarbonate concentration (SBC), total carbonic acid concentration (TCO_2), as well as the level of actual base deficit/excess (ABE) and standard base deficit/excess (SBE). Haemoglobin affinity for oxygen was determined by p50 (oxygen tension in blood at which haemoglobin is saturated with O_2 by 50%) under standard conditions ($p50_{stand}$: temperature 37°C, pH = 7.4, pCO_2 = 40 mm Hg) and actual temperature, pH and pCO_2 ($p50_{act}$). The p50 values obtained and the Hill equation were applied to estimate the position of the oxyhaemoglobin dissociation curve.

2.4. Determination stable end products of NO – nitrates and nitrites

The serum nitric oxide level was evaluated by measuring concentration of stable end products of NO – nitrates and nitrites (NO_3^-/NO_2^-). For blood sampling a 2-ml Heparin Vacuum Tube (VACUETTE®, Austria) was used. Blood plasma was deproteinised using NaOH and zinc sulphate with subsequent reduction of nitrates to nitrites by cadmium granules. The plasma NO_3^-/NO_2^- levels were assessed by the spectrophotometric method with a PV 1251 C spectrophotometer (SOLAR, Belarus) at 540 nm using the Griess reagent [8].

2.5. Statistics

Distribution of the genotypes of the polymorphism studied was evaluated in compliance with the Hardy-Weinberg equilibrium using Pearson's χ^2 test. Statistical analysis was performed by conventional methods using the Statistica 10.0 software. In case of normal distribution of numeric parameters, data were presented as a mean ± standard deviation, and statistical significance of the differences was evaluated by *t*-test for independent samples. In case of non normal distribution, the results were presented as follows: median [25th percentile; 75th percentile], with statistical significance of the differences being determined by the Mann-Whitney test. Correction for multiple testing was done using the method of false discovery rate (FDR) [9]. Differences with FDR < 0.05 (q values) were considered significant and are presented in the text.

3. Results

We evaluated the distribution of the allele frequencies of G894T polymorphism of the NOS3 gene in males (Table 1). The table shows that the distribution of the polymorphism G894T genotypes does not deviate from the Hardy-Weinberg equilibrium, which suggests lack of exogenous influence (mutations, genetic drift, non-random mating) on the genetic structure of the sample.

Upon evaluation of the prevalence of the G894T polymorphism genotypes in the subjects examined, we showed that the homozygous common genotype (GG) was present in 49.1% of the sample, the heterozygous genotype was found in 44.2% of the subjects, and that the frequency of the homozygous recessive genotype (TT) in this group was as low as 6.7%.

Table 2 shows parameters of the oxygen transport function of blood, in healthy volunteers in accordance with the distribution of the allele and genotype frequencies of G894T polymorphism of the NOS3 gene. Investigation of the parameters of the oxygen transport function of

Table 1
Distribution of allele and genotype frequencies of G894T polymorphism of the NOS3 gene in males.

Genotypes			Allele frequency	Hardy-Weinberg equilibrium	
GG	GT	TT	G/T	χ^2	p
81	73	11	0.71/0.29	1.031	0.310

Table 2
Parameters of oxygen transport function of blood related to distribution of allele and genotype frequencies of G894T polymorphism.

	Total n = 165	GG + GT n = 154	GT + TT n = 84	GG n = 81	GT n = 73	TT n = 11
pO_2, mm Hg	22.8 ± 7.6	23.2* ± 7.5	22.2 ± 7.1	23.4* ± 8.0	23.0* ± 7.0	17.0 ± 5.5
CvO_2, mL per 1 L	7.5 ± 3.7	7.7* ± 3.7	7.3 ± 3.5	7.7* ± 3.9	7.6* ± 3.5	3.9 [3.1; 7.3]
SO_2, %	37.9 ± 16.0	38.8* ± 15.9	36.1 ± 15.5	39.8* ± 16.4	37.7* ± 15.3	25.5 ± 13.0
pCO_2, mm Hg	55.7 ± 6.0	55.5 ± 6.0	55.1 [52.8; 58.6]	56.0 ± 6.3	55.0 [52.5; 58.0]	59.1 [52.8; 61.4]
pH, units	7.372 ± 0.031	7.373* ± 0.030	7.370 ± 0.029	7.373* ± 0.032	7.373* ± 0.029	7.351 [7.326; 7.377]
$P50_{stand}$, mm Hg	27.5 ± 2.0	27.7* ± 2.0	27.3 ± 1.9	27.8* ± 2.1	27.5* ± 1.7	25.9 [23.0; 27.6]
$P50_{act}$, mm Hg	27.9 ± 1.7	28.0* ± 1.7	27.6 ± 1.7	28.1* ± 1.9	27.8* ± 1.5	26.3 [24.5; 27.7]

Note: * — differences are statistically significant compared to TT-genotype (FDR-corrected).

blood demonstrated differences related to the distribution of the allele and genotype frequencies of the polymorphism studied. As is evident from the data presented, subjects with the TT-genotype had lower oxygen content in venous blood compared to volunteers with the GT-genotype (3.9 [3.1; 7.3] vs. 7.6 ± 3.5, q = 0.019) and the GG-genotype (3.9 [3.1; 7.3] vs. 7.7 ± 3.9, q = 0.019). A comparison on the recessive model (GG + GT vs. TT) showed that the oxygen content was reduced in volunteers with the TT-genotype (7.7 ± 3.7 vs. 3.9 [3.1; 7.3], q = 0.019).

A comparison of the parameters of oxygen saturation of blood indicated that the saturation of blood in carriers of the TT-genotype turned out to be lower than that in subjects with the GT-genotype (25.5 ± 13.0 vs. 37.7 ± 15.3, q = 0.021) and GG-genotype (25.5 ± 13.0 vs. 39.8 ± 15.3, q = 0.019). In the recessive model, the oxygen saturation of blood was higher as opposed to carriers of the recessive genotype (38.8 ± 15.9 vs. 25.5 ± 13.0, q = 0.019). The oxygen tension in the blood of volunteers with the TT-genotype was lower compared to subjects with the GT-genotype (17.0 ± 5.5 vs. 23.0 ± 7.0, q = 0.019) and the GG-genotype (17.0 ± 5.5 vs. 23.4 ± 8.0, q = 0.019). In their turn, volunteers having a common allele in their genotype (GG + GT) exhibited higher oxygen tension in comparison with bearers of the TT-genotype (23.2 ± 7.5 vs. 17.0, q = 0.019). The blood pH values of the subjects with the common allele (GG + GT) were 0.022 units higher as opposed to the recessive genotype (7.373 ± 0.030 vs. 7.351 [7.326; 7.377], q = 0.039), which is suggestive of some acidic pH shift in this subjects.

It should be noted that the differences in p50 values depended on the genotype. Thus, subjects with the TT-genotype demonstrated lower $p50_{stand}$ parameters compared to volunteers with the GT-genotype (25.9 [23.0; 27.6] vs. 27.5 ± 1.7, q = 0.027) and GG-genotype (25.9 [23.0; 27.6] vs. 27.8 ± 2.1, q = 0.019). A comparison applying the recessive model showed lower $p50_{stand}$ values in subjects with the recessive genotype (27.7 ± 2.0 vs. 25.9 [23.0; 27.6], q = 0.019). As can be seen, the TT-genotype was responsible for higher haemoglobin affinity for oxygen compared to the genotypes with G-allele. A comparison of the $p50_{act}$ values showed similar changes. Subjects with two T-alleles in their genotype, had lower $p50_{act}$ compared to subjects with the GT-genotype (26.3 [24.5; 27.7] vs. 27.8 ± 1.5, q = 0.019) and the GG-genotype (26.3 [24.5; 27.7] vs. 28.1 ± 1.9, q = 0.019). A comparison of this parameter using GG + GT versus TT revealed that carriers of the recessive genotype have lower $p50_{act}$ values (28.0 ± 1.7 vs. 26.3 [24.5; 27.7], q = 0.019). These findings data show that the TT is responsible for the shift of the oxyhaemoglobin dissociation curve to the left (Fig. 1).

The systolic and diastolic pressure values in carriers of the homozygous recessive genotype were significantly higher (Table 3) and the concentration of stable end products of NO was significantly lower (Table 4) as compared to subjects with G allele.

4. Discussion

Studies on the distribution of allele and genotype frequencies of G894T polymorphism have been conducted in different population

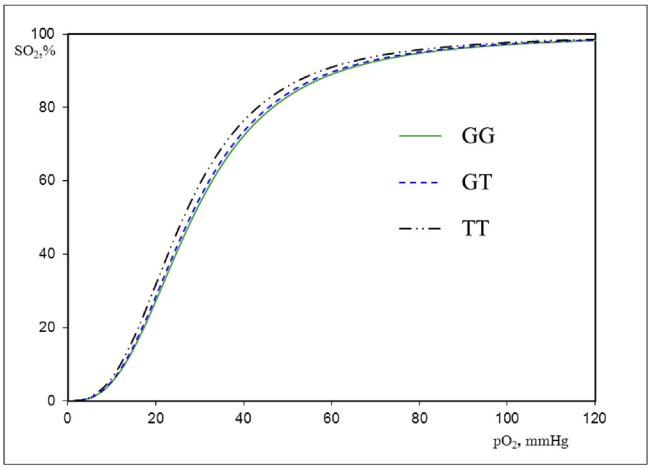

Fig. 1. Oxyhaemoglobin dissociation curve at different genotype of G894T polymorphism of the NOS3 gene.

samples. According to some findings, the prevalence of GG, GT and TT G894T polymorphism in Chinese subjects was 46.2%, 37.8% and 16.0%, respectively, and the frequency of G/T alleles amounted to 65.1%/34.9% [10]. In a study conducted on a homogeneous population of the Caucasian origin (Greeks), Kitsios G.D. and Zintzaras E [11]. showed the distribution of GG, GT and TT G894T polymorphism to be 44.7%, 43.1% and 12.2%, respectively, and the frequency of G/T alleles to be 66.2%/33.8%. The data of our research into the distribution of allele and genotype frequencies of G894T polymorphism are, in general, comparable to the results of similar studies.

Recently, a significant number of papers have been published indicating that G894T polymorphism is associated with development of cardiovascular disorders. For instance, an association between G894T polymorphism and the development of endothelial dysfunction was found [12]. In the work of Guo X. [13], an association between TT-genotype and increased risk of ischaemic stroke compared to genotypes with G-allele was detected, particularly in Asian subjects. A contribution of the TT-genotype to the development of angina was also revealed [14]. It was established that G894T polymorphism was associated with the development of myocardial infarction [15], and an association of T-allele with the development of coronary heart disease was also determined (OR = 1.52, 95% CI = 1.37–1.69) [16].

The above disorders are based on a few pathogenetic factors, with one of which being endothelial dysfunction. It is obvious that the low values for oxygen tension, oxygen content and oxygen saturation of blood, that were found in our study in subjects with the two T-allele genotype of G894T polymorphism, reflected changes in oxygen transport function of blood which might contribute to the development of endothelial dysfunction.

The present study revealed a small (0.022 units) reduction of blood pH values in subjects with the recessive genotype and a simultaneous decrease of the $p50_{stand}$ and $p50_{act}$. Reduction of pH is known to decrease haemoglobin affinity for oxygen and displace the

Table 3
Parameters of body mass index and blood pressure related to distribution of allele and genotype frequencies of G894T polymorphism.

	Total n = 165	GG + GT n = 154	GT + TT n = 84	GG n = 81	GT n = 73	TT n = 11
BMI (kg/m^2)	24 ± 6	24 ± 6	24 ± 7,4	23 ± 3	24 ± 8	24 [21; 27]
SBP (mmHg)	122,0 ± 8,1	121,4* ± 7,6	122,9 ± 8,2	121,1* ± 8,0	121,9* ± 7,1	128,9 ± 11,4
DBP (mmHg)	80,2 ± 6,9	79,6* ± 6,4	80,0 ± 7,9	80,3* ± 5,9	78,7* ± 7,0	90,0 [80,0; 90,0]

Note: * – differences are statistically significant compared to TT-genotype (FDR-corrected).

oxyhaemoglobin dissociation curve to the right. However, human erythrocyte hemoglobin has specific environment, which modifies its main properties [17]. The position of the oxyhaemoglobin dissociation curve in vivo results from a combined interaction of many modulating factors. It is evident that other factors of intraerythrocytic mechanisms regulating blood oxygen-binding properties (pCO_2, 2,3-diphosphoglycerate) are responsible for the compensation of the oxyhaemoglobin dissociation curve shift, observed in our study. At the same time it should be noted that irrespective of the genotype, pH remains within the physiological range and therefore there are no grounds to suggest an acid-base imbalance.

The versatile interaction between NO and haemoglobin should also be mentioned. Under physiological conditions, an increased level of NO is responsible for elevated amount of oxidized and nitrosylated haem, which decreases the total amount of haemoglobin involved in oxygen transport, thus resulting in a reduced general oxygen transport function of blood [18]. The activity of the endothelial NO synthase decreases in the presence of the T-allele of G894T polymorphism [19]. Under conditions of hypoxia, erythrocytic NO synthase may cause a vessel-dilating effect in adjacent to the vessels regions. A significant rise in the activity of erythrocytic NO-synthase with an increase in shear stress to 0.1 Pa was found [20]. NO synthesis in erythrocytes provides effective intracellular signaling and participates in the modification of haemoglobin affinity for oxygen [21]. Our data show that the TT-genotype of G894T polymorphism is associated with a rather low content of O_2 in venous blood. We believe these differences to be of an NO-dependent nature and to be based on the functional state of the endothelium and the activity of the L-arginine–NO pathway.

After binding to NO, the nature of haemoglobin interaction with ligands is changed. Bonaventura C. et al. [5] suggested a model in which haemoglobin affinity for oxygen is related to reduction of nitrites and formation of NO. By binding to the T-quaternary structure of haemoglobin, allosteric anion effectors (chloride, 2,3-diphosphoglycerate, inositol hexaphosphate) change the position of an allosteric balance between the low- (T) and high-affinity (R) structures of this protein, which significantly influences the reactions of haemoglobin with NO and O_2 : the binding of NO with haemoglobin shifts the T-R-balance to the R-side and subsequent binding of oxygen to vacant sites of the tetramer occurs under increased affinity. In turn, the anions, that promote T-state (inositol hexaphosphate), contribute to the development of pentacoordinate geometry of NO-haem, increased haem oxidation and decreased haemoglobin affinity for O_2 [18].

In some of our studies, we have shown participation of NO in the mechanisms of regulation of blood oxygen-binding capacity. Experiments *in vitro* using different concentration relationships showed that when blood is incubated with nitrosocysteine under oxygenation, the $p50_{act}$ was lower by 3.4 ± 0.9 mm Hg ($p < 0.05$) [22]. A shift of the oxyhaemoglobin dissociation curve to the left was observed in hypothermic L-arginine treated rats compared to control animals ($p50_{act}$ = 17.45 ± 0.60 vs. 21.03 ± 0.35 mm Hg ($p < 0.05$)) [3]. In patients with arterial hypertension, endothelial dysfunction promotes impairment of blood oxygen transport processes [23] while the use of products modulating NO synthesis (Nebivololum) normalises parameters of oxygen transport function of blood [24].

Binding of NO to haemoglobin is known to influence its oxygen-binding capacity. The effect of L-arginine seems to result directly from the NO interaction with Hb and to be mediated through an oxygen-dependent mechanism for regulation of NO synthesis [3]. The absence of changes in a number of blood gas values and circulatory buffers (pCO_2, TCO_2, HCO_3^-, SBC, ABE, SBE, ets.) reflects relatively specific influence of G894T polymorphism. The obtained results suggest that the differences in blood oxygen parameters in relation to the genotype are of an NO-dependent nature and can be explained by the functional state of the endothelium and the activity of the L-arginine-NO pathway. On the other hand, the influence of G894T polymorphism alone on the systemic oxygen transport seems to be unlikely. It is obvious that the differences in blood oxygen parameters in different genotypes of G894T polymorphism are due to a number of factors: they arise directly through binding of NO to haemoglobin and indirectly through modulation of allosteric effectors and changes in the functioning of the cardiovascular system. Undoubtedly, other factors also play a role in formation of total oxygen homeostasis. The findings of our study suggest that the differences in oxygen content, pH, and haemoglobin affinity for oxygen are associated with the distribution of allele and genotype frequencies of G894T polymorphism of the NOS3 gene.

5. Conclusion

Thus, the results obtained provide evidence for an association between NOS3 gene G894T polymorphism and blood oxygen transport.

Statement of conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://

Table 4
Plasma stable end products of NO – nitrates and nitrites concentration related to distribution of allele and genotype frequencies of G894T polymorphism.

	Total n = 165	GG + GT n = 154	GT + TT n = 84	GG n = 81	GT n = 73	TT n = 11
NO_3^-/NO_2^- (μmol/L)	10,6 ± 2,4	10,7* ± 2,5	10,3 ± 2,2	10,8* ± 2,6	10,4* [8,9; 11,8]	9,4 [8,8; 9,8]

Note: * – differences are statistically significant compared to TT-genotype (FDR-corrected).

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References

[1] S. Kumar, R.K. Singh, T.R. Bhardwaj, Therapeutic role of nitric oxide as emerging molecule, Biomed. Pharmacother 85 (2017) 182–201.

[2] B.J. Smagghe, J.T. Trent, M.S. Hargrove, NO dioxygenase activity in hemoglobins is ubiquitous in vitro, but limited by reduction in vivo, PLoS One 3 (4) (2008) 2039.

[3] V.V. Zinchuk, L.V. Dorokhina, Blood oxygen transport in rats under hypothermia combined with modification of the L-Arginine-NO pathway, Nitric Oxide 6 (1) (2002) 29–34.

[4] C.C. Helms, M.T. Gladwin, D.B. Kim-Shapiro, Erythrocytes and vascular function: oxygen and nitric oxide, Front. Physiol. 9 (2018) 125.

[5] C. Bonaventura, R. Henkens, A.I. Alayash, S. Banerjee, A.L. Crumbliss, Molecular controls of the oxygenation and redox reactions of haemoglobin, Antioxidants Redox Signal. 18 (17) (2013) 2298–2313.

[6] P.A. Marsden, H.H. Heng, S.W. Scherer, R.J. Stewart, A.V. Hall, X.M. Shi, L.C. Tsui, K.T. Schappert, Structure and chromosomal localization of the human constitutive endothelial nitric oxide synthase gene, J. Biol. Chem. 268 (23) (1993) 17478–17488.

[7] K. Mahmoodi, L. Nasehi, E. Karami, M.S. Soltanpour, Association of nitric oxide levels and endothelial nitric oxide synthase G894T polymorphism with coronary artery disease in the Iranian population, Vasc. Specialist. Int. 32 (3) (2016) 105–112.

[8] N.S. Bryan, M.B. Grisham, Methods to detect nitric oxide and its metabolites in biological samples, Free Radic. Biol. Med. 43 (5) (2007) 645–657.

[9] Y. Benjamini, Y. Hochberg, Controlling the false discovery rate: a practical and powerful approach to multiple testing, J. Roy. Statistic. Soc. 57 (1) (1995) 289–300.

[10] G.P. Zhang, J.N. Sun, J. Wang, H.J. Zhang, C.H. Zhu, L. Lin, Q.H. Li, Z.S. Zhao, X.G. Yu, G.B. Liu, W. Dong, Correlation between polymorphism of endothelial nitric oxide synthase and avascular necrosis of femoral head, Int. J. Clin. Exp. Med. 8 (10) (2015) 18849–18854.

[11] G.D. Kitsios, E. Zintzaras, An NOS3 haplotype is protective against hypertension in a caucasian population, Int. J. Hypertens. (2010 Mar 25) 865031.

[12] V. Saini, M.K. Bhatnagar, J. Bhattacharjee, Association of endothelial dysfunction with endothelin, nitric oxide and eNOS Glu298Asp gene polymorphism in coronary artery disease, Dis. Markers 31 (4) (2011) 215–222.

[13] X. Guo, Endothelial nitric oxide (eNOS) gene G894T and VNTR polymorphisms are closely associated with the risk of ischemic stroke development for Asians: meta-analysis of epidemiological studies, Mol. Biol. Rep. 41 (4) (2014) 2571–2583.

[14] I. García-González, J. Solís-Cárdenas Ade, J.A. Flores-Ocampo, R. Alejos-Mex, L.F. Herrera-Sánchez, L.J. González-Herrera, G894T (NOS3) and G1958A (MTHFD1) gene polymorphisms and risk of ischemic heart disease in Yucatan, Mexico, Clín. Invest. Arterioscler. 27 (2) (2015) 64–73.

[15] J.Q. Luo, J.G. Wen, H.H. Zhou, X.P. Chen, W. Zhang, Endothelial nitric oxide synthase gene G894T polymorphism and myocardial infarction: a meta-analysis of 34 studies involving 21,068 subjects, PLoS One 9 (1) (2014) e: 87196.

[16] K. Zhang, P. Bai, S. Shi, B. Zhou, Y. Wang, Y. Song, L. Rao, L. Zhang, The G894T polymorphism on endothelial nitric oxide synthase gene is associated with increased coronary heart disease among Asia population: evidence from a Meta-analysis, Thromb. Res. 130 (2) (2012) 192–197.

[17] J.F. Storz, Hemoglobin-oxygen affinity in high-altitude vertebrates: is there evidence for an adaptive trend, J. Exp. Biol. 219 (2016) 3190–3203.

[18] A. Fago, A.L. Crumbliss, M.P. Hendrich, L.L. Pearce, J. Peterson, R. Henkens, C. Bonaventura, Oxygen binding to partially nitrosylated haemoglobin, Biochim. Biophys. Acta 1834 (9) (2013) 1894–1900.

[19] M.S. Joshi, C. Mineo, P.W. Shaul, J.A. Bauer, Biochemical consequences of the NOS3 Glu298Asp variation in human endothelium: altered caveolar localization and impaired response to shear, FASEB J. 21 (11) (2007) 2655–2663.

[20] P. Ulker, N. Yaras, O. Yalcin, C. Celik-Ozenci, P.C. Johnson, H.J. Meiselman, O.K. Baskurt, Shear stress activation of nitric oxide synthase and increased nitric oxide levels in human red blood cells, Nitric Oxide 24 (4) (2011) 184–191.

[21] M.M. Cortese-Krott, M. Kelm, Endothelial nitric oxide synthase in red blood cells: key to a new erythrocrine function, Redox Biol. 2 (2014) 251–258.

[22] T.L. Stepuro, V.V. Zinchuk, Nitric oxide effect on the haemoglobin-oxygen affinity, J. Physiol. Pharmacol. 57 (1) (2006) 29–38.

[23] V.V. Zinchuk, T.P. Pronko, M.A. Lis, Blood oxygen transport and endothelial dysfunction in patients with arterial hypertension, Clin. Physiol. Funct. Imag. 24 (4) (2004) 205–211.

[24] T.P. Pronko, V.V. Zinchuk, Effect of nebivolol on blood oxygen transport indices and endothelial dysfunction in patients with arterial hypertension, Clin. Physiol. Funct. Imag. 29 (3) (2009) 170–176.