Supporting Information

Spectroscopy-based characterization of Hb–NO adducts in human red blood cells exposed to NO-donor and endothelium-derived NO

Jakub Dybas[a,b], Piotr Berkowicz[b], Bartosz Proniewski[b], Katarzyna Dziedzic-Kocurek[c], Jan Stanek[c], Malgorzata Baranska[a], Stefan Chlopicki[b,d*], Katarzyna M. Marzec[b,e*]

[a]Faculty of Chemistry, Jagiellonian University, Gronostajowa 2, 30-387 Krakow, Poland.

[b]Jagiellonian Centre for Experimental Therapeutics (JCET), Jagiellonian University, Bobrzynskiego 14, 30-438 Krakow, Poland.

[c]Marian Smoluchowski Institute of Physics, Jagiellonian University, Lojasiewicza 11, 30-348 Krakow, Poland.

[d]Chair of Pharmacology, Jagiellonian University Medical College, Grzegorzecka 16, 31-531 Krakow, Poland.

[e]Centre for Medical Genomics (OMICRON), Jagiellonian University Medical College, Kopernika 7C, 31–034 Krakow, Poland.

Detailed methods description

Isolation of red blood cells from whole human blood

Human blood samples (around 4 cm^3) were collected on heparin as anticoagulant from healthy volunteers on the day of the experiment. Up to one hour after collection, blood was subjected to triple centrifugations (acceleration: 500g; run time: 10 min; RT; braking: 0). The supernatant together with buffy coat were removed by aspiration (after each spinning), and packed RBCs were washed in Ringer–Tris buffer solution. The solution was supplemented with bovine albumin and glucose. The purity of the RBCs fraction, collected after the third centrifugation from the bottom of the tube, was verified. The samples with white blood cells (WBC) count exceeding 200/mm^3 were subjected to another centrifugation. The Ringer–Tris buffer was added to the washed RBCs fraction to obtain the 40% HCT (for BGA, EPR) or 0.1% (for UV–Vis and RRS). The concentration of the heme in the samples was approximately 8 mM and 20 μM, respectively. All of the experiments were conducted within 8 hours after the collection of blood samples.

Chemical and Solutions

PAPA NONOate (PAPA–NO) was purchased from Cayman Chemical (CAS 146672–58–4). This compound spontaneously releases two moles of NO per one mole of compound with a half–life of 15 minutes in 37˚C and 77 minutes at RT. PAPA–NO was firstly dissolved in 0.01M NaOH and then, unless stated otherwise, a proper equivalent of diluted *ex tempore* PAPA–NO solution in Ringer–Tris buffer was added to obtain a proper final concentration (300 μM, 1 mM, 2 mM, 10 mM, 15 mM, 30 mM, 60 mM, 100 mM). As the metHb (metHb–H_2O) standard methemoglobin from bovine blood from Sigma–Aldrich was used (CAS 9008-02-0).

The Ringer–Tris buffer solution was also prepared *ex tempore* with the following composition: 140.5 mM NaCl, 2 mM $CaCl_2$, 4.7 mM KCl, 1.2 mM $MgSO_4$, 21 mM Tris Base, 5.5 mM glucose, and 76 μM bovine albumin. All reagents were dissolved in distilled water and filtered through a 0.22 μm pleated filter; pH was adjusted to 7.35 – 7.45 using 1M hydrochloric acid.

To reduce oxyHb to deoxyHb, the fresh sodium dithionite in amount 6 – 8 mM was used and buffer with RBCs was equilibrated with gaseous N_2 (continuous nitrogen flow across the buffer).

Preparation of RBCs with various oxyHb content

Functional RBCs in buffered solution were prepared at the low (<5%) and high (>90%) oxyHb level. To obtain low oxyHb level of saturation, RBCs were treated with sodium dithionite (2 ± 0.2 mg/ml what respond to 6–8 mM) and placed inside hypoxic chamber under the following parameters: 0.3% O_2, 5% CO_2, 94.7% N_2, 37˚C for one hour. To obtain high oxyHb concentration (>90%), samples were exposed for one hour to atmosphere air (37˚C≈16.3 ml/dl O_2Ct).

Incubations of isolated RBCs with Human Aortic Endothelial Cells

Human Aortic Endothelial Cells (HAEC) were obtained from American Type Culture Collection (ATCC) and used at the 3 – 6 passages after resuscitation of frozen aliquots and regularly tested for Mycoplasma contamination using the MycoAlert Mycoplasma Detection Kit (Lonza). Cells were maintained in T75 culture flasks as adherent monolayer in RPMI 1640 GlutaMAX medium (Gibco, Life Technologies) supplemented with 10% fetal bovine serum (Gibco, Life Technologies), 1 mM sodium pyruvate (Sigma-Aldrich) and antibiotic antimycotic solution (100 units/mL penicillin, 100 µg/mL streptomycin and 25 µg/mL amphotericin B) (Sigma-Aldrich). Cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2. For the each experiment cells were seeded in 6-well plates (seeding density $3x10^{-5}$) and incubated with calcium ionophore (10 µg/ml) for one hour to stimulate production of NO. As negative control HAECs with blocked production of NO by 2h pre-treatment with L–NAME (300 µM) were used. After incubation with calcium ionophore, or L–NAME and calcium ionophore, medium was changed and to HAECs was added suspension of isolated RBCs in amount to obtain 1% HCT. During whole period of incubation time cells were put in rocking motion to mimic semi-flow conditions and strengthen the contact between RBCs and HAECs. After 1, 2 or 6h samples were taken and subjected to UV–Vis and RRS measurements.

Experimental methods

Blood Gas Analysis (BGA) was performed on a Stat Profile pHOx Ultra following the manufacturer's instructions. BGA of RBCs samples were performed in the following time points: 0 (with vehicle – equivalent of Ringer–Tris buffer solution), 5, 15, 30, and 60 minutes after addition of PAPA–NO (300 µM) at 37˚C. Results of the treatment of PAPA–NO with the blood in every oxyHb content group was averaged from four independent experiments (N = 4).

Electron paramagnetic resonance (EPR) spectra were obtained on an EMX EPR Bruker spectrometer, operating at a frequency of 9.45 GHz, with the power of 15.98 mW, modulation amplitude equal to 5 G, time constant of 81.92 s, and sweep time of 20.48 s in the range of 200 G. The number of scans was 30. RBC samples were incubated with PAPA–NO (300 µM) for 15 minutes at 37˚C. Prepared samples were frozen in liquid nitrogen, transferred into the dewar, and subjected to the analysis.

Absorption spectra (UV–Vis) were performed on a Perkin Elmer double beam spectrophotometer Lambda 950 in the range of 350 to 700 nm using a cuvette of 1 cm path length. All samples were prepared in the same way as described in Section 2.3 and diluted 1:1000 (v:v). RBC samples were incubated with different PAPA–NO concentration (300 µM, 1mM, 2mM, 10mM, 15mM, 30mM, 60 mM, 100mM) up to around 270 minutes at RT and UV–Vis spectra were recorded for specific time intervals. Samples were measured for each PAPA–NO concentration for closed and opened cuvettes (in order to mimic experimental condition for Raman measurements). In case of closed cuvettes samples were additionally sealed by mineral oil layer on the top of the solution. In case of opened cuvettes samples were exposed to air environment during whole time of measurements.

Mössbauer Spectroscopy measurements were performed in the transmission mode with a 50mCi ^{57}Co source in a Rh matrix, using a *Wissel* spectrometer. The velocity of the spectrometer was calibrated using a standard Fe foil at room temperature. RBCs samples with the high oxyHb content were incubated with PAPA–NO (300 µM) for 60 minutes at RT. Prepared samples were placed in a plexi containers, 19 mm in diameter and 6 mm thick, frozen in liquid nitrogen, transferred into the dewar, further placed in the Oxford cryostat and subjected to the Mössbauer measurements at 80 K. The RBC samples contained ^{57}Fe isotope of its natural abundance. Data were numerically evaluated with the use of WinNorms-for-Igor Software (WaveMetrics Inc.). Two types of RBCs cell were examined: 1) high oxyHb content, treated as the control sample and 2) high oxyHb content, incubated with PAPA–NO. In both cases, control UV–Vis measurements were performed.

Resonance Raman Spectroscopy (RRS) measurements of isolated, viable RBCs were recorded using a WITec confocal CRM alpha 300 Raman microscope. The spectrometer was equipped with an air–cooled solid–state lasers operating at 488 nm and a proper CCD detector that was cooled to –60°C. The lasers were coupled to the microscope via optical fibre with a diameter of 50 µm in case of 488 nm excitation line. A water immersive Nikon Fluor (60x/1.00W) objective was used. The spectral resolution was equal to 3 cm^{-1}. The monochromator of the spectrometer was calibrated using a radiation spectrum from a calibrated xenon lamp (Witec UV–light source). Additionally, the standard alignment procedure (single-point calibration) was performed before measurement with the use of the Raman scattering line produced by a silicon plate (520.5 cm^{-1}). RBC samples were diluted 1:1000 (v:v) with buffer solution and put into a glass bottom dish with CaF_2 plate. RBCs were first measured in Ringer–Tris buffer solution equilibrated with air (high oxyHb). For low oxyHb content, all Raman measurements were carried out in the OKOlab microscope cooling/heating incubator (H101–UP chamber) placed on microscope scan table. For low oxyHb, nitrogen was introduced to the gas chamber to replace air, and additionally treated with sodium dithionite (2 ± 0.2 mg/ml) under constant flow of N_2. Measurements were started from around 60 minutes after PAPA–NO addition in RT and were recorded up to 4h.

Raman spectra of functional RBCs at different concentrations of oxyHb were averaged from 600 – 1 200 single Raman spectra obtained from different RBCs at each condition. Each single spectrum was acquired with the integration time of 3 s and 1 scan. The laser intensity in the focus spot was equal to 9 µW (as

previously reported, higher laser power for such high energy excitation can cause the photo/thermal dissociation of oxyHb [9]) for 488 nm excitation and 100 µW in order to visualize laser-induced dissociated oxyHb and HbNO adducts. Raman measurements and data analysis were performed using WITec software (WITec Project Plus 2.10), Opus 7.2, and Origin 9.1. All averaged Raman spectra were pre–processed (cosmic spike removal, smoothing (9), and background subtraction) and normalised. Obtained results were repetitive for N = 4 (four different blood samples, for around 600 – 1200 single RBCs per each sample).

Figures

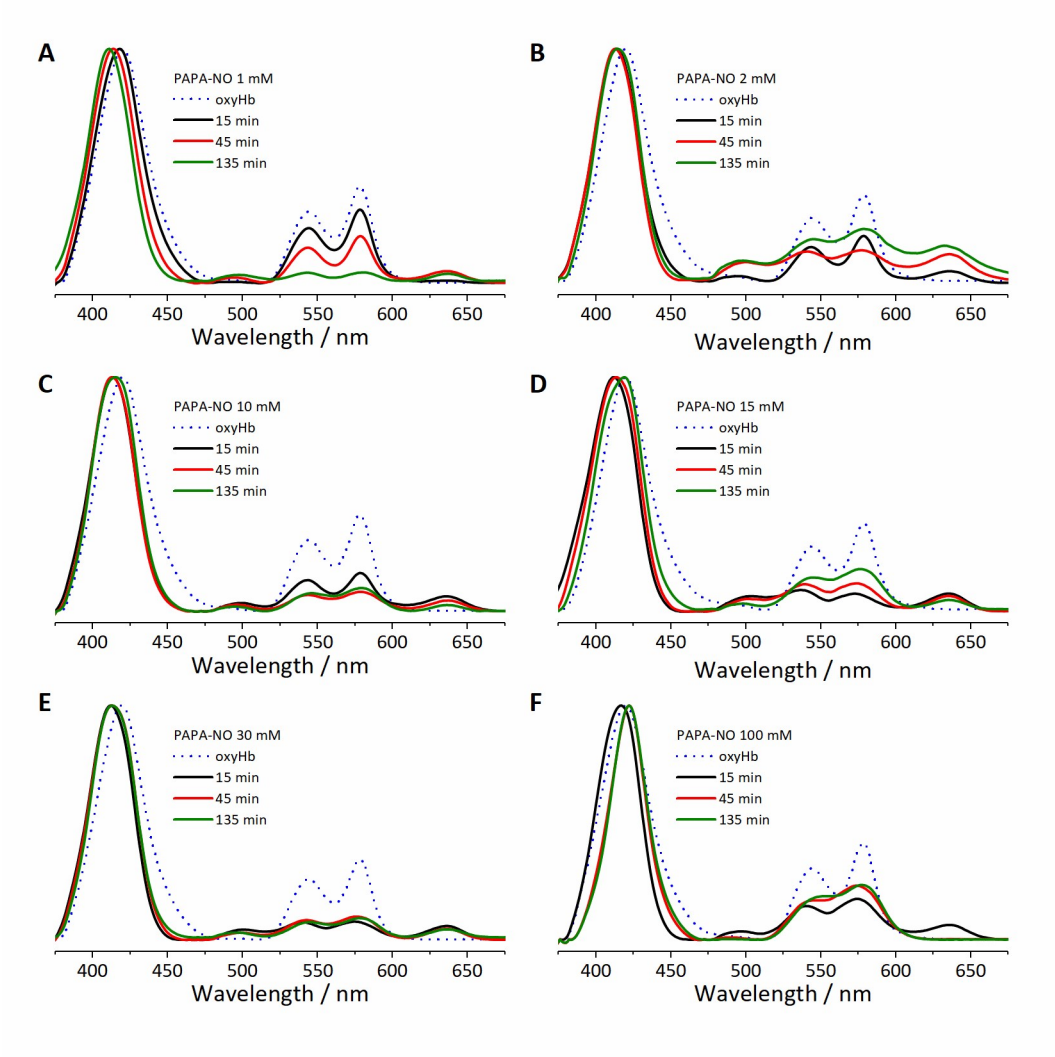

Figure SM1. Kinetics of changes in UV–Vis absorption spectra after addition of various concentrations of PAPA–NO (1 – 100 mM, $t_{1/2}$ = 77 minutes at RT) to isolated human RBCs with high oxyHb content for open cuvettes.

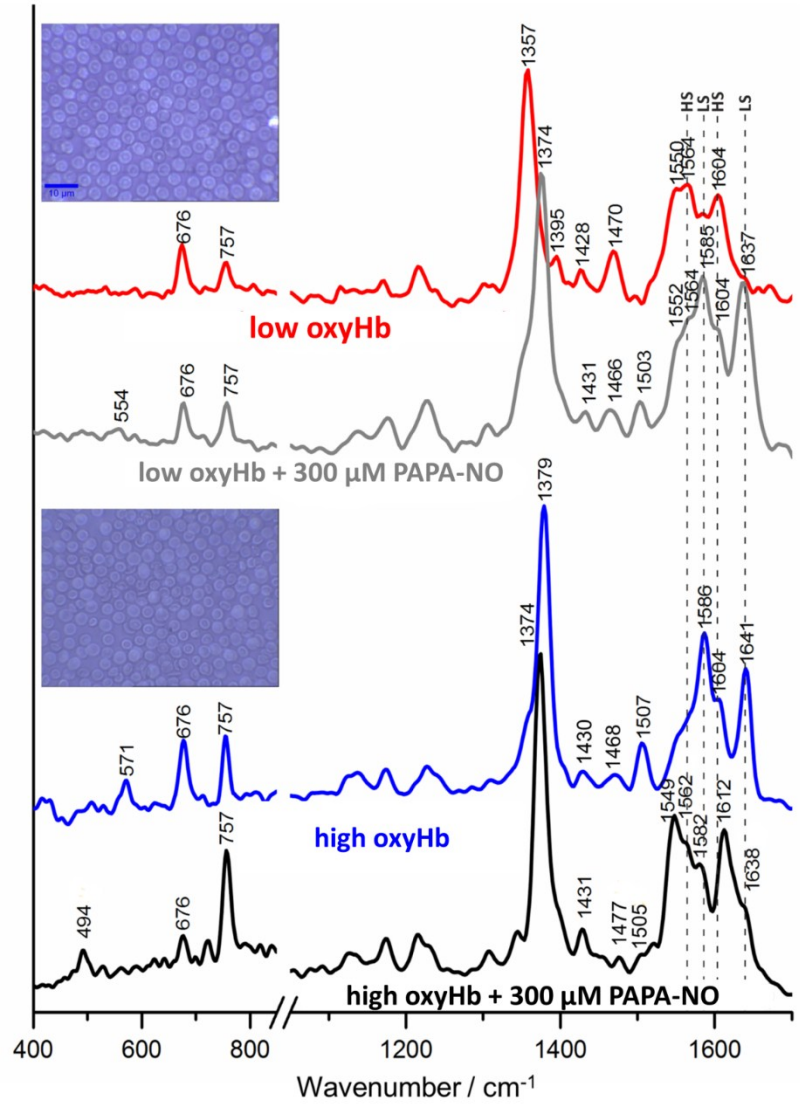

Figure SM2. The average Raman spectra taken from isolated human RBCs before and after exposition to PAPA–NO (300 µM) in experimental conditions of low (<5%) and high (>90%) levels of oxyHb saturation. Each average spectrum was obtained from 1200 single spectra, each of which was obtained from a different RBC, recorded in period of time 60-240 minutes after each sample preparation. Spectra were obtained with the use of 488 nm laser excitation and with the laser intensity in the focus spot approximately equal to 9 µW. The insert images present the homogenous monolayer of RBCs representative for experimental conditions of low and high levels of oxyHb saturation in which spectra were collected directly from the pure population of isolated RBCs maintained in physiological conditions.

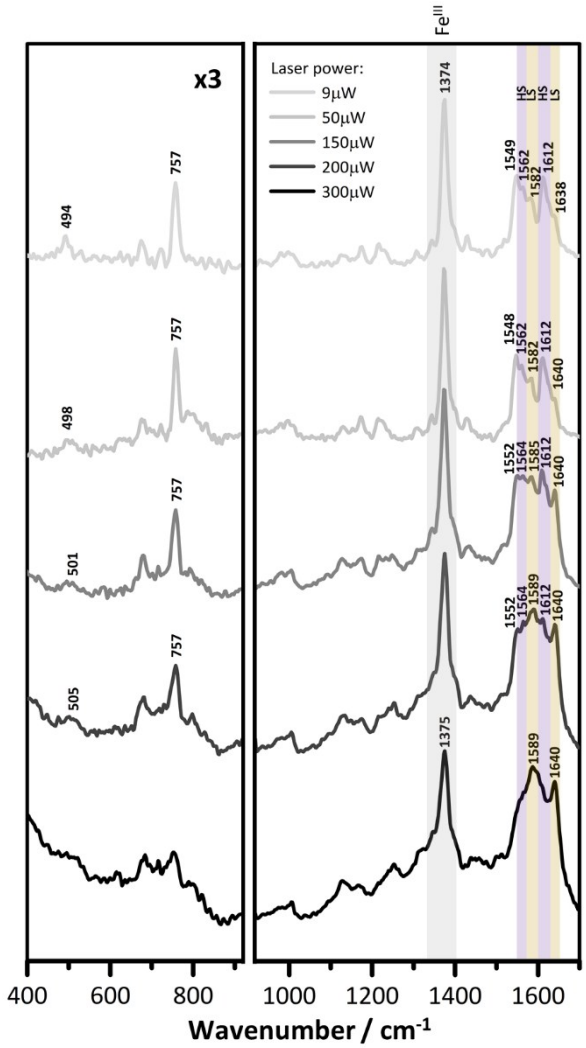

Figure SM3. The averaged RRS spectra of high level of oxyHb saturation treated with PAPA–NO (300 μM) obtained with the use of 488 nm laser excitation with the laser intensity in the focus spot equal to approximately 9, 50, 150, 200 and 300 μW. Each of the spectrum was averaged from 600 single spectra of different RBCs.

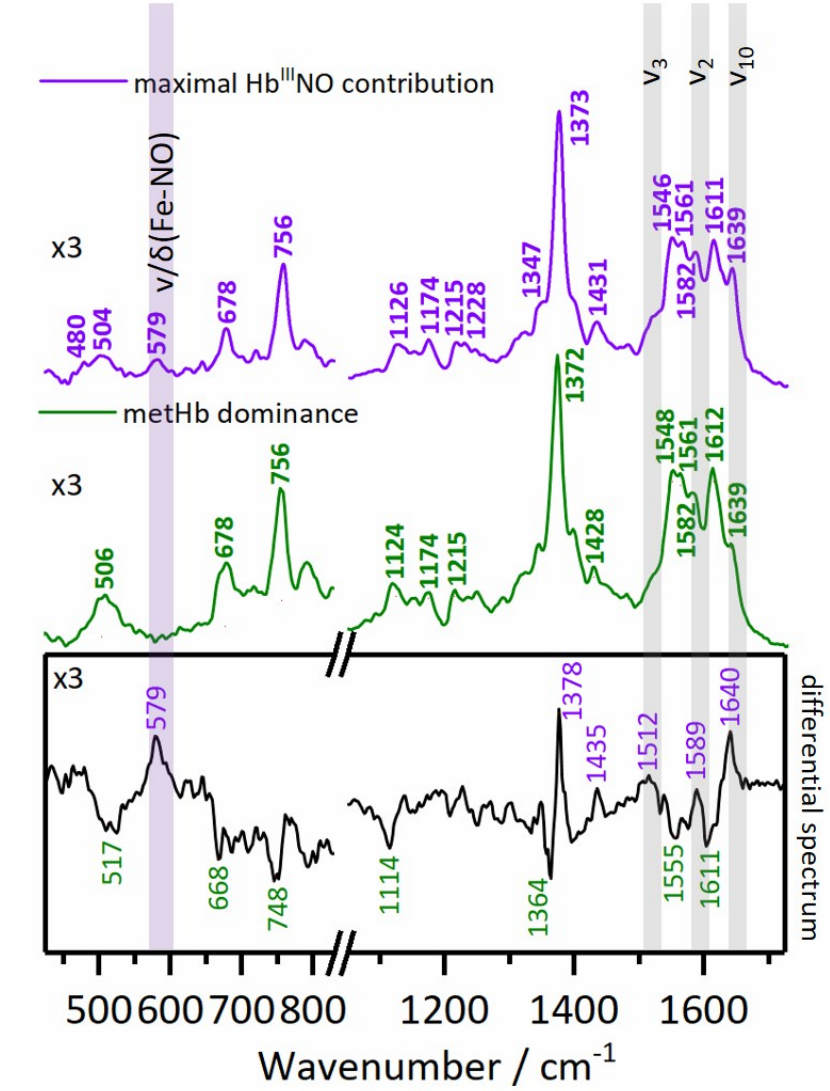

Figure SM4. The averaged RRS spectra of maximal $Hb^{III}NO$ contribution (purple line) and metHb dominance (green line) with the differential spectrum (black line). All spectra were obtained with the use of 488 nm laser excitation with the laser intensity in the focus spot equal to approximately 9 μW. Each of the spectrum was averaged from 600 single spectra of different RBCs from 3 independent biological experiments (200 spectra per experiment).

Tables

Table SM1. Summary of the average BGA parameters obtained from the control RBCs (without PAPA–NO donor, 0 min), and after the addition of nitric oxide donor, PAPA–NO (300 μM) in 5, 15, 30, and 60 minutes of experiment. Measurements of high oxyHb were carried out in the air atmosphere, whereas middle and low oxyHb were investigated in the hypoxic chamber.

Samples	*Parameter*	*0 min*	*5 min*	*15 min*	*30 min*	*60 min*
Low OxyHb (n=4)	O_2Hb [%]	2.9 ± 0.94	3.1 ± 0.36	3.3 ± 0.22	3.2 ± 0.05	3.4 ± 0.21
	COHb [%]	1.6 ± 0.21	3.0 ± 0.21	3.7 ± 0.14	4.0 ± 0.10	4.0 ± 0.07
	MetHb [%]	0.9 ± 0.29	2.1 ± 0.52	2.3 ± 0.40	2.0 ± 0.36	1.7 ± 0.27
	HHb [%]	94.6 ± 0.88	91.8 ± 1.01	90.6 ± 0.58	90.9 ± 0.37	90.9 ± 0.25
	SO_2 [%]	3.0 ± 0.97	3.2 ± 0.39	3.6 ± 0.23	3.4 ± 0.05	3.6 ± 0.22
	O_2Ct [ml/dl]	0.5 ± 0.17	0.5 ± 0.09	0.5 ± 0.04	0.5 ± 0.02	0.5 ± 0.00
High OxyHb (n=4)	O_2Hb [%]	92.4 ± 2.91	91.1 ± 2.95	90.7 ± 2.07	89.2 ± 1.39	86.0 ± 1.46
	COHb [%]	1.6 ± 0.17	1.6 ± 0.22	1.5 ± 0.21	1.5 ± 0.22	1.4 ± 0.17
	MetHb [%]	0.6 ± 0.05	1.9 ± 0.24	3.6 ± 0.41	5.6 ± 0.48	8.8 ± 0.43
	HHb [%]	5.5 ± 3.07	5.7 ± 3.08	4.2 ± 2.59	3.7 ± 1.93	3.9 ± 1.94
	SO_2 [%]	94.4 ± 3.12	94.1 ± 3.15	95.6 ± 2.67	96.0 ± 2.01	95.7 ± 2.11
	O_2Ct [ml/dl]	16.3 ± 0.57	15.4 ± 0.73	16.0 ± 0.36	14.6 ± 0.59	12.9 ± 0.58

******O_2Hb – oxyhemoglobin (oxyHb), COHb – carboxyhemoglobin ($Hb^{II}CO$), metHb – methemoglobin, HHb – deoxyhemoglobin, SO_2 – the level of O_2 saturation, O_2Ct – oxygen content (total amount of oxygen in the blood)*