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# Changes in lymphocyte properties after employment of the combination of carbamylation and oxidative stress, an in vitro study

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ABSTRACT

It is well known that oxidative stress and carbamylation alter macromolecule properties and functions.
We evaluated the influence of sodium cyanate (NaOCN) and the combination of cyanate and hydrogen peroxide ($H_2O_2$) on nonenzymatic antioxidant capacity (NEAC), total thiols, reduced glutathione (GSH) and hydroperoxide level in mononuclear blood cells (MNCs). We also examined plasma membrane properties of MNCs using the spin labeling method in EPR spectroscopy (electron paramagnetic resonance spectroscopy).
We showed that MNCs are resistant to cyanate treatment up to a concentration of 2 mM (survival test). On the other hand, a significant loss of antioxidant defense of cells, e.g. NEAC upon NaOCN, $H_2O_2$ and the combination of cyanate and hydrogen peroxide was observed. Carbamylation slightly decreased GSH and the free thiol level, but $H_2O_2$ and its combination with NaOCN lead to a decrease in their amounts. A markedly higher level of hydroperoxides was only observed in the cells treated with $H_2O_2$.
We found a significant decrease in lipid membrane fluidity at the depth of 12th and 16th carbon atoms of fatty acids in lymphocytes treated with cyanate or $H_2O_2$. The combination of both substances acted synergistically and induced profound changes in comparison to cyanate and hydrogen peroxide used alone.


## 1. Introduction

Carbamylation of proteins and oxidation of cellular components during oxidative stress occur in pathology of many diseases [e.g., diabetes mellitus, atherosclerosis, chronic renal failure (CRF)] (Jaisson and Gillery, 2010; Jaisson et al., 2011).

Carbamylation is the posttranslational modification of proteins resulting from the nonenzymatic reaction between isocyanic acid and specific free functional groups, especially amino groups. Isocyanic acid is a very reactive form of cyanate derived from the spontaneous decomposition of urea ( Wynckler et al., 2000; Jaisson et al., 2011). In the body, an aqueous environment, urea and cyanate occur in equilibrium (99:1) (Wynckler et al., 2000).

It has been demonstrated that in patients with chronic renal failure (CRF) the concentration of urea is 5–7 times higher than in healthy individuals (Malyszko et al., 2006; Selvaraj et al., 2002; Wyncler et al., 2000; Trepanier et al., 1996). Moreover, it has been demonstrated that in CRF hemodialysis (HD) patients the concentration of cyanate before HD increases up to 150 nM and in comparison to healthy volunteers is about three times greater (Nilsson et al., 1996).

Another serious problem occurring in CRF patients is oxidative stress and microinflammation (Jaber et al., 2001). These problems may contribute to progression of renal disease, and can be associated with higher mortality of patients. It has been suggested that two main reasons of an increase of oxidative stress in hemodialysis patients exist. The first relates to increased production of reactive oxygen species as a result of activation of immune cells in response to a small biocompatibility of the dialysis membrane (Hörl, 2002). The second reason is the low selectivity of the dialysis membrane which may result in the removal of uremic toxins and low molecular weight antioxidants from the blood of uremic patients. It has been observed that in hemodialyzed patients, the antioxidant capacity of plasma before HD is higher than in healthy volunteers but after a dialysis session it drops significantly below the level of normal value (Pieniazek et al., 2002a, 2002b).

Several studies have demonstrated synergism between oxidative stress and inflammatory response. Hydrogen peroxide as a metabolic oxidant can activate the NF-kB pathway, leading to synthesis of proinflammatory cytokines and resulting in amplification of the inflammatory cascade (Libetta et al., 2011).

A small number of works associated with the combined action of carbamylation and oxidative stress available in the literature prompted us to study the effect of both factors on the properties of MNCs. The aim of this study was to evaluate the in vitro influence of carbamylation and the combination of carbamylation and oxidative stress on isolated human peripheral blood mononuclear cells. We determined the effect

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of the combination of carbamylation and oxidation on oxidative stress parameters such as nonenzymatic antioxidant capacity, total thiols, reduced glutathione and peroxide level. Additionally, we examined also the plasma membrane after incubation of cells with cyanate, hydrogen peroxide and their combination by measuring lipid membrane fluidity using EPR spectroscopy.

## 2. Material and methods

### 2.1. Chemicals

2,3-bis[2-Methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide inner salt (XTT), Phenazine methosulfate (PMS), 2,4,6-tripyridyl-s-triazine (TPTZ), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 5-doxyl-stearic acid (5-DS), 12-doxyl-stearic acid (12-DS), 16-doxyl-stearic acid (16-DS), 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), o-phthalaldehyde (OPA), and N-ethylmaleimide (NEM) were obtained from the Sigma-Aldrich company. The RPMI 1640 bicarbonate medium, fetal bovine serum (FBS), penicillin and streptomycin were from PAA (Germany). All other chemicals were purchased from POCH S.A. (Gliwice, Poland) if not otherwise indicated.

All dishes necessary for cell culture were obtained from the NUNC company.

### 2.2. Peripheral blood mononuclear cells (MNCs)

All experiments were carried out on human normal peripheral blood mononuclear cells (MNCs) isolated from the blood obtained from the Blood Bank in Lodz, Poland (each time, from leukocyte layers yielded separately from blood of different healthy volunteers). Therefore, every single experiment was performed on cells from one donor — n-numbers represent cells from different individuals.

Cell isolation was performed by centrifugation of blood in the density gradient of Histopaque 1077 (300 *g* for 30 min at 22 °C). The cells were washed two times with phosphate buffered saline (PBS). The MNCs were grown in the RPMI medium supplemented with 10% inactivated fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml) in a 37 °C humidified 5% $CO_2$ incubator.

Isolated cells were plated on a sterile dish ($2 \times 10^6$ cells/ml) (5 ml each sample) then sodium cyanate (NaOCN) to the final concentration of 1 mM or 2 mM was added and incubated in a 37 °C humidified 5% $CO_2$ incubator for 23 h. At the end of this period, hydrogen peroxide was added to a final concentration of 100 μM and left for 1 h of incubation. The collected cells were washed with phosphate buffered saline (pH 7.4) (PBS) and used for membrane lipid fluidity determination. For measurements of reduced glutathione and thiol group concentration as well as antioxidant activity, the cells were lysed in a buffer containing 10 mM Tris, 1 mM EDTA, 0.1 M NaCl and 0.01% Triton X-100 (about $1 \times 10^7$ cells in 0.1 ml buffer).

### 2.3. Cell survival

The survival of blood mononuclear cells after treatment with sodium cyanate was estimated by the standard microplate XTT colorimetric method. Isolated blood mononuclear cells ($15 \times 10^4$ cells/well) were seeded on 96-well microplates in the growth medium with a wide range of sodium cyanate concentrations (0–10,000 μM) for 24 h and incubated at 37 °C in a humidified 5% $CO_2$ incubator. At the end of the exposure time the medium with NaOCN was replaced with a fresh one and the cells were grown for additional 24 h. At this time point the XTT test was performed. Freshly prepared XTT (0.05 ml, to the final concentration of 1 mg/ml) with PMS (to the final concentration of 0.017 mg/ml) was added to each well of the microplates. Subsequently, the microplates were incubated for 4 h. Absorbance was measured at 495 nm using a microplate reader (BioTek).

2,3-bis[2-Methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide inner salt (XTT) was used to quantify the metabolically active living cells. The mitochondrial dehydrogenases of viable cells reduce the tetrazolium ring of XTT, yielding an orange formazan derivative.

The obtained values of absorbance of the reaction product were presented as a percentage, taking the value of absorbance of the control as 100%.

### 2.4. Nonenzymatic antioxidant capacity

The nonenzymatic antioxidant capacity (NEAC) of MNCs was measured using two different methods: TPTZ and DPPH.

The spectrophotometric method is based on reduction of the ferric tripyridyltriazine [Fe(III)-TPTZ] complex to ferrous [Fe(II)] at a low pH. The iron complex [Fe(III)-TPTZ] reduced by antioxidants has an intense blue color with absorption maximum at 593 nm (Benzie and Strain, 1996). The samples of lysed cells (0.05 ml) were mixed with a working reagent (300 mM acetate buffer (pH 3.6); 10 mM TPTZ in 40 mM HCl; 20 mM $FeCl_3$ freshly prepared in a volume ratio (10:1:1)) (0.1 ml) and incubated at room temperature for 30 min. At the end of incubation the absorbance was measured.

The electron paramagnetic resonance (EPR) method is based on reduction of the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical. In the presence of antioxidants, loss of EPR signal of 1,1-diphenyl-2-picrylhydrazyl (DPPH) is observed. The loss of EPR signal is inversely proportional to the concentration of antioxidants (Pieniazek et al., 2002a, 2002b).

The samples of lysed cells (0.01 ml) were then mixed with DPPH (300 μM in methanol) (0.04 ml) and incubated for 30 min at room temperature. At the end of incubation the samples were centrifuged and the intensity of EPR signal in the supernatant was measured.

In both methods the antioxidant activity of MNCs was expressed as Trolox equivalents calculated on the basis of the calibration curve (in nmol/mg protein).

EPR spectra were performed on the Bruker ESP 300 E spectrometer at room temperature (22 + 2 °C), operating at a microwave frequency of 9.73 GHz. The instrumental settings were as follows: center field set at 3480 G, range of 80 G, with a 100 kHz modulation frequency and modulation amplitude of 1.01.

### 2.5. Free thiol group concentration

The quantification of free thiol groups was based on reaction with Ellman's reagent (5,5′-dithiobis-(2-nitrobenzoic acid); DTNB). Upon reaction with free thiol groups 2-nitro-5-thiobenzoate (NTB), optically active at 412 nm, is released (Ellman, 1959). The samples of lysed cells (0.015 ml) were then mixed with 10 mM phosphate buffer (pH 8.0) containing 0.5% SDS (0.235 ml) and the absorbance at 412 nm was measured ($A_0$). Then the DTNB reagent was added to the samples (final concentration of 0.1 mM) (0.025 ml) and they were incubated for 1 h at 37 °C. After this time the absorbance of the samples was again measured $A_1$. $\Delta A = A_1 - A_0$ for calculation of the thiol group concentration was used.

The concentration of thiol groups was calculated based on the calibration curve for different concentrations of reduced glutathione and expressed as nmol/mg protein.

### 2.6. Glutathione

The reduced glutathione (GSH) concentration was determined using the fluorimetric method with o-phthalaldehyde (OPA) (Senft et al., 2000). The reaction product of OPA and GSH has high fluorescence quantum yields. The mononuclear cell lysate (0.01 ml) was mixed with redox quenching buffer with trichloroacetic acid (RQB-TCA (20 mM HCl, 5 mM DTPA, 10 mM ascorbic acid); 5% TCA)