Clinical research

Systemic oxidoreductive balance and vascular function in individuals without clinical manifestation of atherosclerosis

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Abstract

Introduction: Endothelial dysfunction is recognized as the earliest disorder in the development of atherosclerosis, in the pathogenesis of which oxidative stress plays a crucial role. The aim of this study was to determine the relationships between non-invasive parameters of vascular dysfunction and oxidative stress.

Material and methods: Forty-eight individuals without clinical manifestation of atherosclerosis were studied. The plasma concentrations of the following were determined in all 48 subjects: retinol, ascorbic acid, α-tocopherol and uric acid, as well as the products of oxidative DNA damage repair: 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) in blood leukocytes and urine, and 8-oxo-7,8-dihydroguanine (8-oxoGua) in urine. The following parameters of vascular dysfunction were also examined: flow- (FMD) and nitroglycerin- (NMD) mediated dilatation of the brachial artery, pulse pressure (PP), distensibility coefficient (DC), pulsation (PI) and resistance (RI) index, carotid intima-media thickness (cIMT), and ankle-brachial index (ABI).

Results: Individuals with an FMD value of ≥ 8.8% had significantly higher blood concentrations of antioxidative vitamins and lower concentrations of 8-oxodG in their urine and blood leukocytes than their counterparts. Blood concentration of alpha-tocopherol or ascorbic acid positively correlated with FMD, PI, RI, DC and ABI and negatively with PP and cIMT. The reverse was the case for 8-oxodG in urine and leukocytes. In multiple regression analysis, markers of oxidative DNA damage positively determined the variance in PP and ABI.

Conclusions: In persons without clinical manifestation of atherosclerosis, oxidative stress was an independent factor associated with vascular wall dysfunction, and a better predictor than smoking and blood concentrations of glucose, lipids and creatinine.

Key words: oxidative stress, DNA oxidative damage markers, vascular function, flow-mediated dilatation, intima-media thickness, ankle-brachial index.
Introduction

Endothelial dysfunction plays a crucial role in atherosclerosis development and progression. It is an endpoint effect of all known atherosclerosis risk factors (e.g. smoking, hypertension, diabetes and hyperlipidemia) and promotes atherogenesis through its influence on vasodilation mechanisms, the stimulation of arterial thrombosis, and the migration and proliferation of vascular wall cells [1, 2]. The most frequent cause of endothelial dysfunction is oxidative stress, which is an effect of the activation of redox signaling pathways through the influence of proatherogenic conditions, including high levels of low-density lipoprotein (LDL) and its oxidized form (ox-LDL) [3].

Endothelial dysfunction is recognized as a biomarker of early and subclinical injury of the vascular wall and assists in the cardiovascular risk stratification of patients with various comorbidities [4–7]. Finally, endothelial dysfunction leads to functional or morphological abnormality of the vascular wall, signs of which justify an increase in the intensity of the control of atherosclerosis risk factors. The most frequently used markers in the diagnosis of arterial wall deterioration, vessel performance and/or its hemodynamic consequences are: (a) increase in carotid intima-media thickness (cIMT), (b) decrease in ankle-brachial index (ABI); (c) brachial artery flow-mediated dilatation (FMD), which reflects vasorelaxation dependent on endothelial function, and endogenous nitric oxide release; this test is sometimes complemented by sublingual nitroglycerine application (nitroglycerine-mediated dilatation – NMD), which assesses the possibility of vasodilatation independent of endothelial function (endothelium-independent vasmotion), and makes it possible to evaluate the entire vasomotor reserve of the smooth muscles that form the vessel wall [8–13]; (d) tonometry of peripheral arteries (digital pulse amplitude tonometry or PAT) to calculate the passive hyperemia index, augmentation index and reactive hyperemia in response to ischemia (RH-PAT) [4, 11–13]; (e) arterial stiffness examination based on pulse pressure (PP) measurement (the difference between systolic and diastolic blood pressure, whereby an amplitude greater than 55 mm Hg shows increased cardiovascular risk), pulse wave shape and propagation (pulse wave velocity – PWV) registered in the proximal and distal points of the circulatory system; (f) arterial distensibility coefficient (DC), which is expressed as the difference between systolic and diastolic diameters of the common carotid artery divided by the product of values for the diastolic artery diameter and pulse pressure; (g) pulsatility and resistance indexes, increased values of which represent higher risk for atherosclerosis [4–13].

Although oxidative stress is directly involved in atherosclerosis development and progression, only a few studies on the relationships between circulating parameters of oxidative stress and vascular wall function are available. We hypothesized that a decreased level of antioxidant in blood and increased concentration of markers of oxidative tissue damage might help to identify individuals at risk of early atherosclerosis development, who seem to be most likely to benefit from therapeutic intervention. Therefore, we performed an investigation with the aim of assessing the relationships between selected parameters of vascular wall function and markers of systemic oxidoreductive balance in persons without clinical manifestation of atherosclerosis.

Material and methods

We studied 48 individuals without a history of atherosclerosis manifestation. The inclusion criteria were as follows: no history of clinical atherosclerosis manifestation, in the following forms: acute coronary syndrome, myocardial infarction, stroke, transient ischemic attack, intermittent claudication, aortic aneurysm or other vascular diseases; and aged over 18 and having the ability to give informed consent for participation in the study. The exclusion criteria were as follows: symptoms or history of any vascular diseases; hypertension, diabetes mellitus (as an equivalent of vascular diseases); chronic pharmacotherapy, intake of diet supplements which could affect oxidoreductive balance; the inability to perform a treadmill stress test; and history or physical signs of inflammatory diseases or neoplasm.

The following were undertaken for all the subjects who qualified for the study: medical history (symptoms, past history of diseases, use of stimulants, daily intake of vegetables and fruit, and use of diet supplements and vitamins); and physical examination and basic laboratory investigations (blood morphology, determinations of creatinine, sodium, potassium, glucose, alanine aminotransferase, C-reactive protein, total, LDL, and HDL cholesterol, and triglycerides).

Parameters of oxidoreductive balance

Detailed examinations of oxidoreductive balance were performed, as previously described [14–16]:

- in plasma: the concentration of high-sensitivity C-reactive protein (hs-CRP), as well as retinol (vitamin A), ascorbic acid (vitamin C), tocopherol (vitamin E), and uric acid;
- in blood leukocyte DNA: 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG);
- urinary excretion of 8-oxo-7,8-dihydroguanine (8-oxoGua) and 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG);
• antioxidative status (AS) – defined as the geometric mean concentration of antioxidant vitamins determined;
• prooxidative status (PS) – defined as the geometric mean concentration of DNA damage products determined;
• redox index: defined as the ratio of PS to AS.

Parameters of vascular dysfunction
The following parameters of vascular wall function were calculated [7–13]:
• brachial artery FMD, with the measurement of changes in internal brachial artery systolic diameter after 5 min of arm compression using a cuff at a pressure that is 30 mm Hg greater than systolic blood pressure; this was calculated according to the following formula: FMD(%) = 100% × (systolic diameter after arm ischemia – systolic diameter before arm ischemia)/systolic diameter before arm ischemia; this parameter reflects endothelium-dependent vasodilatation;
• NMD of brachial artery one minute after sublingual (sl.) application of 0.4 mg nitroglycerine (NTG) in spray form; this was calculated in accordance with the formula: NMD(%) = 100% × (systolic diameter after sl. NTG application – systolic diameter before sl. NTG application)/systolic diameter before sl. NTG application; this parameter reflects endothelium-independent vasodilatation;
• ABI, which was calculated as a quotient of the systolic blood pressures measured at the ankle and in the upper arm, presented as the lower of two values from both sides.

Bioethics
The study protocol was approved by the local Bioethical Committee, and the study was conducted in compliance with the Declaration of Helsinki for medical research. All study participants signed informed consent for participation in the study.

Statistical analysis
Statistical analysis was conducted using licensed versions of statistical software Statistica (a data analysis software system), StatSoft, Inc. (2015), version 12. The normal distribution of the study variables was checked using the Shapiro-Wilk test. The results were mainly presented as the mean ± standard deviation or n, %. The statistical significance of the differences between groups was verified using Student’s t-test and the χ² test. The statistical significance level was set at a p-value < 0.05. Pearson’s correlations were determined. Stepwise backward multiple regression (using the General Regression Models module of Statistica) was applied to evaluate the relationships between the values of the respective parameters of vascular wall function and the parameters of oxidoreductive balance. Subjects’ age, gender, and the values of the above-mentioned parameters of oxidoreductive balance and laboratory tests were selected as the independent variables, and the above-listed parameters of vascular wall function were used as the dependent variables.

Results
The participants of our study were divided into two groups in relation to the median FMD value (Table I). Subjects with better endothelial function (FMD ≥ 8.88%) had higher plasma concentrations of ascorbic acid, retinol and α-tocopherol, as well as significantly lower concentrations of 8-oxodG in their urine and leukocytes than their counterparts. Moreover, they were younger, less prevalently male; had lower creatinine blood concentration, and had similar blood concentrations of plasma lipids and glucose. Greater endothelium-dependent vasodilatation was also related to
Table I. Clinical and biochemical parameters in patients divided in relation to the median value of flow-mediated brachial artery dilatation (FMD)

| Parameter                                      | FMD ≥ 8.88% | FMD < 8.88% | P-value |
|------------------------------------------------|-------------|-------------|---------|
| Age [years]                                    | 54.88 ±4.28 | 58.04 ±4.76 | 0.02    |
| Male gender, n (%)                             | 10 (42)     | 18 (75)     | < 0.01  |
| BMI [kg/m²]                                    | 27.28 ±4.14 | 27.83 ±3.11 | 0.61    |
| Current smoker, n (%)                          | 5 (21)      | 3 (12.5)    | 0.51    |
| Plasma hs-CRP [mg/l]                           | 2.02 ±1.68  | 1.48 ±1.01  | 0.14    |
| Plasma ascorbic acid [µM]                      | 44.58 ±21.08| 32.57 ±18.08| 0.04    |
| Plasma retinol [µM]                            | 2.93 ±0.45  | 2.53 ±0.76  | 0.03    |
| Plasma α-tocopherol [µM]                       | 35.96 ±6.67 | 31.83 ±5.51 | 0.02    |
| Plasma uric acid [µM]                          | 355.54 ±53.61| 356.69 ±69.30| 0.24   |
| 8-oxoGua in urine [nmol/mmol creatinine]       | 7.05 ±2.84  | 10.26 ±7.73 | 0.062   |
| 8-oxodG in urine [nmol/mmol creatinine]        | 2.18 ±0.83  | 2.82 ±1.12  | 0.027   |
| 8-oxodG in leukocytes (8-oxodG/10⁶ dG)         | 5.97 ±1.49  | 7.29 ±2.38  | 0.03    |
| Creatinine [mg/dl]                             | 1.01 ±0.13  | 1.12 ±0.16  | 0.01    |
| Blood glucose concentration [mg/dl]            | 98.64 ±18.69| 94.52 ±17.58| 0.44    |
| Total cholesterol [mg/dl]                      | 222.28 ±44.43| 220.00 ±29.88| 0.84   |
| HDL cholesterol [mg/dl]                        | 59.76 ±15.20| 58.22 ±9.72 | 0.68    |
| LDL cholesterol [mg/dl]                        | 138.60 ±34.27| 135.00 ±26.44| 0.69   |
| Triglycerides [mg/dl]                          | 118.48 ±36.53| 118.43 ±47.78| 0.99   |
| FMD                                            | 9.28 ±5.55  | 3.52 ±2.99  | < 0.001 |
| NMD                                            | 10.61 ±2.99 | 9.98 ±4.19  | 0.51    |
| Pulse pressure [mm Hg]                         | 43.60 ±7.97 | 48.13 ±9.73 | 0.084   |
| Mean difference between systolic and diastolic internal diameter of carotid arteries | 0.39 ±0.21 | 0.40 ±0.17 | 0.89    |
| Carotid wall thickness (cWT)                   | 0.23 ±0.04  | 0.25 ±0.06  | 0.12    |
| Pulsatility index (PI)                         | 1.65 ±0.081 | 1.73 ±0.10  | < 0.01  |
| Resistance index (RI)                         | 0.79 ±0.06  | 0.84 ±0.07  | < 0.01  |
| Distensibility coefficient (DC)                | 20.85 ±11.64| 15.80 ±6.73 | 0.08    |
| Elasticity modulus (EM)                        | 51.20 ±9.42 | 41.25 ±8.03 | < 0.001 |
| Mean cIMT [mm]                                 | 0.76 ±0.14  | 0.86 ±0.18  | 0.04    |
| Mean ABI                                       | 1.21 ±0.17  | 1.21 ±0.19  | 0.98    |

Data presented as mean ± standard deviation (SD) and number (n, %) of qualitative variables. The statistical significance level was set at a p-value < 0.05; 8-oxoGua in urine – concentration of 8-oxoguanine in urine calculated in relation to the concentration of creatinine, 8-oxodG in urine – concentration of 8-oxo-7,8-dihydro-2′-deoxyguanosine in urine calculated in relation to the concentration of creatinine, 8-oxodG in leukocytes – 8-oxo-7,8-dihydro-2′-deoxyguanosine content in leukocytes, BMI – body mass index, hs-CRP – plasma concentration of C-reactive protein determined using the high-sensitivity method, HDL – high-density lipoprotein, LDL – low-density lipoprotein, FMD – flow-mediated dilatation of brachial artery, NMD – nitroglycerin-mediated dilatation of brachial artery, cWT – relative carotid wall thickness, cIMT – carotid intima-media thickness, ABI – ankle-brachial index.
greater values of EM, and lower PI, RI, PP (not significant) and cIMT (Table I). Similar split analyses in relation to the median value were performed for NMD, PP, PI, RI, cWT, DC, EM, cIMT and ABI. However, the analyses did not show statistically significant differences in the oxidoreductive balance parameters between the groups of subjects with respect to the aforementioned parameters (data not presented in detail).

In univariate analysis, the plasma concentrations of antioxidative substances, such as ascorbic acid, α-tocopherol, AS and PS, were positively correlated with parameters of arterial wall function (e.g. FMD) or hemodynamics in leg arteries (ABI), and negatively with indices of arterial stiffness or narrowing (e.g. PI, RI and cIMT) (Table II). On the other hand, an inverse pattern of relationships was found between the above-mentioned parameters (e.g. PR, PI, RI, DC, EM, ABI) and the oxidative stress indices (e.g. DNA damage markers). To determine which of the factors that were significant in univariate analysis were independent factors that affected vascular wall function, we performed stepwise backward multiple regression analysis (Table III). We found that the FMD value was independently determined only by the participants’ age and gender, and that none of the factors analyzed appeared to be an independent predictor of DC or EM values. However, the other parameters of vascular wall function were independently related to parameters of oxidoreductive balance. PP independently correlated with 8-oxodG concentration in leukocytes; PI and RI were significantly related to ascorbic acid blood concentration; and cIMT and ABI values were independently related to AS and PS, respectively (Table III). However, the adjusted determination coefficients of these multiple regression equations were not high (9–59%).

**Discussion**

In our study, we examined the associations between biomarkers of antioxidative balance and parameters of vascular wall function in individuals without clinical manifestation of atherosclerosis. Subjects with better endothelial function, expressed by an FMD value ≥ 8.8%, had higher plasma concentrations of vitamins A, C and E and lower concentrations of DNA injury products in urine and blood leukocytes compared to their counterparts (Table I). It should be underlined that, apart from being younger, being female, and having a lower blood creatinine concentration, none of the factors determined, including smoking status, blood glucose and plasma lipids, significantly discriminated individuals with respect to differences in endothelial function. Moreover, we also found some weak but statistically significant correlations between numerous parameters of vessel...
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Table III. Stepwise backward multiple regression analysis for respective parameters determining vascular wall function

| Dependent/independent variable | β     | Standard error for β | t (48) | P-value |
|--------------------------------|-------|----------------------|--------|---------|
| FMD: R² = 0.30; adjusted R² = 0.26; F = 0.54; p = 0.02 |       |                      |        |         |
| Constant                       | –2.49 | 0.02                 |        |         |
| Age                            | –0.43 | 0.14                 | –3.02  | < 0.001 |
| Female gender                  | 0.38  | 0.14                 | 2.71   | 0.01    |
| PP: R² = 0.14; adjusted R² = 0.11; F = 0.57; p = 0.02 |       |                      |        |         |
| Constant                       | 12.39 | < 0.001              |        |         |
| 8-oxodG in leukocytes          | 0.37  | 0.15                 | 2.38   | 0.022   |
| PI: R² = 0.61; adjusted R² = 0.39; F = 27.49; p < 0.001 |       |                      |        |         |
| Constant                       | –3.88 | < 0.001              |        |         |
| Female gender                  | 0.52  | 0.11                 | 4.58   | < 0.001 |
| Ascorbic acid                  | –0.33 | 0.11                 | –2.90  | 0.01    |
| RI: R² = 0.39; adjusted R² = 0.57; F = 25.71; p < 0.001 |       |                      |        |         |
| Constant                       | –3.95 | < 0.001              |        |         |
| Female gender                  | 0.52  | 0.12                 | 4.41   | < 0.001 |
| Ascorbic acid                  | –0.31 | 0.12                 | –2.67  | 0.01    |
| cIMT: R² = 0.11; adjusted R² = 0.09; F = 5.9; p = 0.02 |       |                      |        |         |
| Constant                       | 9.88  | < 0.001              |        |         |
| AS                             | –0.34 | 0.14                 | –2.43  | 0.02    |
| ABI: R² = 0.11; adjusted R² = 0.09; F = 4.65; p = 0.04 |       |                      |        |         |
| Constant                       | 28.01 | < 0.001              |        |         |
| PS                             | –0.33 | 0.15                 | –2.16  | 0.04    |

Data presented as R and p-value; FMD – flow-mediated dilatation of brachial artery, PP – pulse pressure, PI – pulsatility index, RI – resistance index, cIMT – carotid intima-media thickness, AS – antioxidative status defined as the geometric mean concentration of antioxidant vitamins, ABI – ankle-brachial index, PS – prooxidative status defined as the geometric mean concentration of DNA damage products.

wall function and oxidative stress biomarkers (Table II), which confirmed the vasoprotective activity of antioxidants and the harmful effect of oxidative stress, markers of which, such as products of DNA damage, were related to worse vascular wall function. In multifactorial analysis, parameters of oxidoreductive balance independently determined the variances of PP, PI, RI, cIMT and ABI, although the equations obtained had low determination coefficient values (Table III).

The results obtained by us corroborate previously published data. However, the majority of the previous research concerned the effects of dietary antioxidant intake or supplementation rather than blood concentration of antioxidative vitamins. In a study by Joris and Mensink [17], the supplementation of high doses of vitamin E (tocopherol) increased FMD, whereas treatment with vitamin D did not appear to have this effect [17]. A study by Ives et al. [18] on patients with chronic obstructive lung disease found that a mixture of vitamin C, vitamin E, and α-lipoic acid significantly improved FMD, and this effect was negatively correlated with baseline plasma vitamin E concentration. Improvement of endothelial function after antioxidant supplementation was also observed by Khan et al. [19] following consumption of blackcurrant juice with high levels of vitamin C content and polyphenols, by Johnson et al. [20] as a result of acute vitamin C supplementation, and by Uzun et al. [21] after supplementation with vitamin C alone or its combination with vitamin E. A meta-analysis by Ashor et al. [22] revealed age-dependent improvement of endothelial function in investigations in which vitamin C (500–2000 mg/day) and vitamin E (300–1800 IU/
The association between circulating vitamin C and risk of stroke was also suggested a significant inverse relationship with respect to differences in endothelial function. Nagao et al. [27] found that serum α-tocopherol level in Japanese women was inversely associated with arterial stiffness. Rimoldi et al. [26] found a significant correlation between changes in plasma ascorbic acid level and FMD after 4 weeks of oral supplementation with vitamins C (1 g/day) and E (400 IU/day) or a placebo. Moreover, in other studies, the blood concentrations of oxidative stress parameters were associated with the prevalence of major adverse cardiovascular events, which are recognized as the clinical endpoints of endothelial dysfunction. Nagao et al. [27] also found that serum α-tocopherol level in Japanese women was inversely associated with total stroke mortality, and a meta-analysis by Chen et al. [28] also suggested a significant inverse relationship between circulating vitamin C and risk of stroke.

Unfortunately, as with most authors, we could not avoid some methodological shortcomings that could have influenced the strength of the deductions based on our results, in spite of obtaining statistically significant effects. Firstly, the number of subjects included in the study was relatively small. Secondly, the inclusion criteria were based on participants' medical history and their medical documentation, but not on invasive examinations such as coronaryography or angiography of the peripheral arteries. On the other hand, carotid artery ultrasound with cIMT determination and ABI are recognized as useful screening tools for the early diagnosis of vascular injury and the stratification of patients' risk [7]. Thirdly, the values of the parameters of oxidoreductive balance are dependent on many factors, which are sometimes difficult to identify in clinical examination but which may be potential sources of laboratory errors and mistakes in interpretation. In our study we excluded patients with neoplastic and inflammatory diseases and individuals taking synthetic vitamins, as well as patients with chronic diseases and elevated blood concentrations of hs-CRP and CRP.

In conclusion, in patients without clinical manifestation of atherosclerosis, oxidative stress, de-
terminated on the basis of parameters detected in plasma (antioxidative vitamins), blood leukocytes, and urine, was an independent factor associated with vascular wall dysfunction and a better predictor than smoking and blood concentrations of glucose, lipids and creatinine. Further studies are needed to evaluate the role of oxidative stress parameters as biomarkers of endothelial dysfunction, which is recognized as the earliest stage of atherosclerosis development.

Conflict of interest
The authors declare no conflict of interest.

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