Research article

On the potential increase of the oxidative stress status in patients with abdominal aortic aneurysm

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Background: Abdominal aortic aneurysm (AAA) is a major cause of preventable deaths in older patients. Oxidative stress has been suggested to play a key role in the pathogenesis of AAA. However, only few studies have been conducted to evaluate the blood oxidative stress status of AAA patients.

Methods and results: Twenty seven AAA patients (mean age of 70 years) divided into two groups according to AAA size (≤50 or >50 mm) were compared with an age-matched group of 18 healthy subjects. Antioxidants (vitamins C and E, β-carotene, glutathione, thiols, and ubiquinone), trace elements (selenium, copper, zinc, and copper/zinc ratio) and markers of oxidative damage to lipids (lipid peroxides, antibodies against oxidized patients, and isoprostanes) were measured in each subject. The comparison of the three groups by ordinal logistic regression showed a significant decrease of the plasma levels of vitamin C ($P = 0.011$), α-tocopherol ($P = 0.016$) but not when corrected for cholesterol values, β-carotene ($P = 0.0096$), ubiquinone ($P = 0.014$), zinc ($P = 0.0035$), and of selenium ($P = 0.0038$), as AAA size increased. By contrast, specific markers of lipid peroxidation such as the Cu/Zn ratio ($P = 0.046$) and to a lesser extent isoprostanes ($P = 0.052$) increased.

Conclusion: The present study emphasizes the potential role of the oxidative stress in AAA disease and suggests that an antioxidant therapy could be of interest to delay AAA progression.

Keywords: Antioxidants, Trace elements, Lipid peroxidation, Cardiovascular research, Oxidative stress status

Introduction

In Western countries, abdominal aortic aneurysm (AAA) ruptures are responsible for about 1.3% deaths among 65–85 years old men. Despite considerable advances in pharmacological treatment and medical technology, mortality after conventional surgery and endovascular treatment still ranges between 1.2 and 6%. AAA is characterized by a localized structural wall deterioration leading to progressive aortic dilatation. Besides other known pathophysiological processes, the infiltration of inflammatory leukocytes promotes aneurismal remodelling,[1,2] in the sense that, once activated in the tissue, leukocytes release metalloproteinases (MMPs) and elastase that contribute to wall degradation.[3] Further, increased oxidative stress (OS) due to leukocytes respiratory burst leads to a higher production of reactive oxygen species (ROS), including free radicals (superoxide anion and hydroxyl radical) and hydrogen peroxide, in the extracellular medium.[4] ROS rapidly enhance lipid peroxidation, also known to trigger atherosclerosis development.[5] Red blood cell (RBC) haemagglutination in the intra-luminal thrombus (ILT) releases free haemoglobin, which in turn generates large quantities of ROS and ultimately develops oxidative stress in AAA patients.[6,7] Development of OS through inducible nitric oxide synthesis (iNOS) induction and endothelium dysfunction may also contribute to AAA pathologic features.[8–10] To regulate ROS production and combat their deleterious effect, the organism responds with a large and complex battery of substances including enzymes, proteins, iron chelators, low molecular weight compounds (glutathione and ubiquinone), trace elements (copper, zinc, and selenium), and antioxidants (vitamins A, C, E, carotenoids, and polyphenols).[11] Recently, Jones defined OS as an imbalance

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DOI 10.1179/1351000212Y.0000000012

between oxidants (e.g. free radical species derived from oxygen) and antioxidants in favour of oxidants, leading to a disruption of redox signalling and/or molecular damage.[12]

To the best of our knowledge, only a few studies investigated the presence and the role of ROS in AAA patients;[13–22] moreover these studies always focused on one or two markers of the oxidative stress and hence provided only partial information on the potential relationship between OS status and AAA size. The present study was specifically designed to identify antioxidants, trace elements, and markers of lipid peroxidation as blood biomarkers of the oxidative stress in AAA patients.

Materials and methods

Study design

Twenty seven patients with a mean age of 70 years and a documented AAA by CT scans underwent a comprehensive check-up of oxidative stress biomarkers in blood. Patients with connective tissue disorders were excluded from the study. A sample of 18 age-matched presumably healthy volunteers (mean age: 68 years), in whom AAA was excluded based on abdominal US examination, was constituted to serve as the control group. The day before the examination visit, subjects fasted for at least 12 hours and were not allowed to drink fruit juice and to perform physical activity. The day of the visit, information including age, height, weight, arterial blood pressure, smoking habits, pathologies, intake of drugs and antioxidants, and consumption of fruits and vegetable was collected by means of a home-made questionnaire. The study protocol was approved by the University Hospital Ethics Committee for medical research. All patients and volunteers received written information about the goal of the study and signed an informed consent form prior to participation.

Oxidative stress biomarkers

Antioxidants determination

For vitamin C, 0.5 ml plasma (ethylenediaminetetraacetic acid (EDTA) blood) was immediately transferred to ice-cold tubes containing 0.5 ml of 10% metaphosphoric acid. The whole mixture was frozen on dry ice. Analyses were performed on the day of blood collection by a spectrophotometric method using the reduction of 2,6-dichlorophenolindophenol (Perkin Elmer Lambda 40, Norwalk, USA).[23] Plasma vitamin E (α- and γ-tocopherols), β-carotene, and ubiquinone were assayed by HPLC procedure (Alliance, Waters, USA) coupled with a diode array detector (PDA 2996, Waters, USA)[24] using Chromsytems kits (32 000, 34 000, and 68 000). Thiol proteins were detected according to Ellman's method.[25] The GSH/GSSG ratio (GSH = reduced glutathione and GSSG = oxidized glutathione), as marker of oxidative stress, was determined in whole EDTA blood by the GSH/GSSG-412 kit (Bioxytech, Oxis international Inc., Portland, WA, USA).

Trace element analysis

The plasma (heparinized blood) levels of selenium, copper, and zinc were determined by inductively coupled plasma-mass spectroscopy.[26]

Oxidative damage to lipids

The analysis of lipid peroxides (ROOH) as marker of oxidative damage to lipids was performed in plasma (EDTA blood) with the commercial kit (Oxystat, Biomedica Gruppe, Wien, Austria). Briefly, the peroxide (–OOH) concentration was determined spectrophotometrically by reaction of the biological peroxides with peroxidase and a subsequent colour reaction using 3,3′,5,5′-tetramethylbenzidine as substrate. The titre in free antibodies (IgG) against oxidized low-density lipoprotein (Ab-Ox-LDL) was assessed with a commercial enzyme immunoassay (Biomedica Gruppe) using Cu^{2+} oxidized LDL as antigen.

Isoprostanes

The detection of F2α-isoprostanes in urine samples was performed by liquid chromatography mass spectroscopy analysis as previously described.[27]

Neutrophil activation

Upon activation, neutrophils release ROS and myeloperoxidase (MPO) into the extracellular medium. Plasma MPO, as indirect marker of ROS production, was assessed using MPO Elisa Kit purchased by ImmunDiagnostik, Bensheim, Germany.

Statistical analysis

Results were expressed as mean and standard deviation (SD) for quantitative measurements and as numbers and percentages for categorical attributes. For some variables, a log-transform was applied to normalize their distribution. To analyse biomarkers of the oxidative stress, the 27 AAA patients were further divided into two groups according to their largest antero-posterior and/or transverse aneurismal diameter (d) measured by CT: group I: $d \leq 50$ mm ($n = 15$) and group II: $d > 50$ mm ($n = 12$). The control group ($d = 0$ mm) and the two AAA groups were then compared by ordinal logistic regression analysis. Results were considered significant at the 5% critical level ($P < 0.05$). All calculations were done by means of the SAS (version 9.2 for Windows) statistical package.

Results

Table 1 summarizes the demographic, biometric, medical, and dietary characteristics of control subjects

Table 1 Demographic, biometric, medical, and dietary characteristics of controls and AAA patients

Variable	Control group ($n = 18$)	AAA patients ($n = 27$)
Gender		
Men	11 (61)	23 (85)
Women	7 (39)	4 (15)
Age (years)	67.4 ± 7.0	69.4 ± 8.0
Smoking		
No	15 (83)	17 (63)
Yes	3 (17)	10 (27)
Height (cm)	171 ± 4.8	172 ± 6.8
Weight (kg)	78.6 ± 8.7	78.5 ± 14.9
BMI (kg/m^2)	26.9 ± 3.2	26.4 ± 5.1
Systolic blood pressure (mmHg)	125.7 ± 10.1	134.5 ±19.6
Diastolic blood pressure (mmHg)	76 ± 10.1	77 ± 7.13
Fruit intake (servings)	1.8 ± 0.5	2.1 ± 0.4
Vegetables intake (servings)	1.7 ± 0.6	1.7 ± 0.4
Diabetes		
No	17 (94)	24 (88)
Yes	1 (6)	3 12)
Hypertension		
No	11 (61)	12 (44)
Yes	7 (39)	15 (56)
Hyperlipidaemia		
No	12 (67)	10 (58)
Yes	6 (33)	17 (42)
Acute myocardial infarction		
No	18 (100)	22 (81)
Yes	0 (0)	5 (19)
Pectoris angina		
No	18 (100)	24 (88)
Yes	0 (0)	3 (12)
Medicamention		
Aspirin		
No	15 (83)	6 (22)
Yes	3 (17)	21 (78)
β-Blockers		
No	11 (62)	17 (63)
Yes	7 (38)	10 (27)
Statins		
No	15 (83)	9 (34)
Yes	3 (17)	18 (66)

and AAA patients. The proportions of men and smokers were slightly higher in the AAA than in age-matched control subjects. No significant difference was observed for height, weight, body mass index (BMI), arterial blood pressure, and fruits and vegetables intake. Presence of diabetes, hyperlipidaemia, acute myocardial infarction, and angina pectoris was comparable in both groups. The majority of AAA patients were under aspirin and statin medication in contrast to control subjects.

When considering biomarkers of the oxidative stress, ordinal logistic regression indicated that plasma levels of vitamin C ($P = 0.011$), β-carotene ($P = 0.0096$), ubiquinone ($P = 0.014$), zinc ($P = 0.0035$), and selenium ($P = 0.0038$) significantly decreased with AAA size (Table 2). Specifically, with respect to concentrations observed in the control group, vitamin C levels decreased by 16 and 33% in groups I and II, respectively. For β-carotene, a drop of 31 and 62% was observed, whereas for ubiquinone the decrease amounted 17 and 32.2%, respectively. Levels of α-tocopherol significantly decreased according to AAA size ($P = 0.016$) but remained constant when divided by cholesterol ($P = 0.71$). Selenium levels were lowered by, respectively, 10 and 24%. Zinc concentrations fell from 0.79 ± 0.14 mg/l in the control group to 0.73 ± 0.10 mg/l in AAA group I and to 0.63 ± 0.13 in AAA group II. By contrast, the Cu/Zn ratio significantly increased from 1.14 ± 0.21 to 1.24 ± 0.38 and 1.43 ± 0.42 ($P = 0.046$). The levels of isoprostanes rose by, respectively, 18 and 66% in the AAA groups when compared with the control group but without reaching statistical significance ($P = 0.052$). All other parameters investigated in this study were not found to be related to AAA size. Finally, it is worth mentioning that age, smoking, fruits, and vegetables consumption did not influence the circulating concentrations of vitamin C, β-carotene, and the other parameters (data not shown).

Discussion

Although current evidence on the role of oxidative stress in the development of AAA mostly emerges from tissue analysis, there is only limited information available on the actual OS status in AAA patients. In 1996, we reported that plasma levels of vitamin E (α-tocopherol) were reduced in AAA patients.[19] Here we confirmed a significant decrease of about 20% of vitamin E levels, probably linked to the concomitant depleted cholesterol concentrations. Indeed, 66% of AAA patients (18 of 27) and only 18% of control subjects (3 of 17) were treated for hyperlipidaemia with statins, whereas cholesterol and liposoluble α-tocopherol levels are known to be strongly correlated. Further, we clearly demonstrated that serum biomarkers of the antioxidant status (vitamin C, β-carotene, ubiquinone, selenium, and zinc) were significantly altered in AAA patients, in particular as AAA size increases. Besides its own free radical activity, vitamin C also acts in synergy with α-tocopherol to limit the lipid peroxidation process. In our study, vitamin C levels were markedly lower in AAA patients but remained on average within reference values (6.2–18.8 μg/ml). Whereas many studies have emphasized that subjects with vitamin C plasma levels <6 μg/ml were at higher risk of cardiovascular events,[28] we found that among patients with AAA size >56 mm, vitamin C levels averaged 6.8 ± 2.95 μg/ml, close to the 6 μg/ml cutoff level (data not shown).

Among all parameters investigated in this study, β-carotene evidenced the most significant decrease (62%) in AAA patients. In agreement with Gey[29] who reported that β-carotene levels <0.22 mg/l were associated with an increased risk of developing

Table 2 Mean plasma levels of oxidative stress biomarkers in control subjects and AAA patients classified according to the size of aneurysm (cutoff 50 mm)

Variable	Reference values	Control group ($n = 18$)	AAA ≤50 mm ($n = 15$)	AAA >50 mm ($n = 12$)	*P*-value**
Vitamin C (μg/ml)	6.2–18.8	10.9 ± 3.85	9.15 ± 2.73	7.35 ± 3.16	0.011
α-Tocopherol (μg/ml)	8.6–19.2	14.5 ± 3.34	12.4 ± 3.14	11.6 ± 2.91	0.016
γ-Tocopherol (μg/ml)	0.28–2.42	0.81 ± 0.38	0.92 ± 0.49	0.66 ± 0.29	0.50
Cholesterol (g/l)	1.2–1.9	2.04 ± 0.35	1.79 ± 0.54	1.62 ± 0.46	0.020
α-Tocopherol /cholesterol (mg/g)	4.4–7	7.16 ± 1.44	7.10 ± 1.31	7.38 ± 1.64	0.71
β-Carotene (mg/l)	0.05–0.68	0.29 ± 0.17	0.20 ± 0.18	0.11 ± 0.054	0.0096
Thiol proteins (μM)	310–523	311 ± 38	337 ± 45.7	312 ± 41.0	0.48
Ubiquinone (mg/l)	0.3–1.39	0.84 ± 0.32	0.70 ± 0.23	0.57 ± 0.21	0.014
Copper (mg/l)	0.8–1.20	0.88 ± 0.12	0.90 ± 0.26	0.90 ± 0.32	0.79
Zinc (mg/l)	0.7–1.20	0.79 ± 0.14	0.73 ± 0.10	0.63 ± 0.13	0.0035
Copper/zinc ratio	1–1.17	1.14 ± 0.21	1.24 ± 0.38	1.43 ± 0.42	0.046
Selenium (μg/l)	94–130	92.7 ± 16.4	83.8 ± 18.3	70.4 ± 21.0	0.0038
Lipid peroxides (μM)*	0–432	520 ± 228	574 ± 360	563 ± 307	0.82
Oxidized LDL (ng/ml)*	0–500	756 ± 964	205 ± 244	264 ± 220	0.062
Antibodies against oxidized LDL (UI/l)*	200–600	263 ± 282	255 ± 308	148 ± 87.5	0.32
Isoprostanes (ng/ml)	NA	1.01 ± 0.66	1.19 ± 0.64	1.68 ± 0.89	0.052
Paraoxonase (ng/ml)*	NA	105 ± 61.1	127 ± 89.0	136 ± 72.2	0.26
Total glutathione (μM)*	717–1110	852 ± 203	960 ± 148	922 ± 209	0.20
Oxidized glutathione (μM)*	1.17–5.32	1.01 ± 0.67	4.58 ± 11.0	4.93 ± 13.3	0.33
Glutathione peroxidase (UI/g Hb)*	20–58	51.5 ± 9.97	51.5 ± 11.5	51.0 ± 10.6	0.91
Myeloperoxidase (ng/ml)*	0–55	22.0 ± 24.4	53.4 ± 76.6	48.8 ± 95.9	0.28

*Log-transform applied to the variable.
***P*-value from ordinal logistic regression.
NA = not available.

cardiovascular diseases and cancer, we found that the mean plasma β-carotene level observed in patients with AAA size >50 mm was 0.11 ± 0.054 mg/l, notably below this threshold.

Ubiquinone is not only a membrane stabilizer and important antioxidant but also an essential cofactor for mitochondrial energy production. We found ubiquinone levels significantly decreases, reaching a 32.2% drop in patients with large AAA sizes. It is difficult though to establish a direct link between such a drop and the presence of increased OS, mainly because 18 of the 27 patients were taking statins which are 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors and act by decreasing mevalonate levels, a precursor for both cholesterol and ubiquinone synthesis.[30] In this study, cholesterol levels significantly decreased according to AAA groups concomitantly with those of ubiquinone, their ratio (ubiquinone/cholesterol) however remained unchanged (0.41) in all groups (data not shown).

Selenium is the determinant cofactor of glutathione peroxidase, a physiologically important lipid peroxide-decomposing enzyme. Witkowska *et al.*[20] reported a negative correlation between serum selenium and the diameter of AAA ($r = -0.38$). We confirmed this finding since selenium dropped by 10% in AAA patients of group I and by 24% in those of group II. In the latter group, the plasma level averaged 70.4 ± 21 μg/ml, way below the lower reference value of 94 μg/ml. In AAA patients, the levels of GPx, a seleno-dependent enzyme, remained unchanged when compared with those of the control group. This was quite unexpected since a previous study[14] reported a decrease of GPx activity in AAA tissues.

In the copper–zinc superoxide dismutase which catalyses the dismutation of superoxide anion, Cu plays a major role in the catalytic cycle while zinc stabilizes the protein structure. The concentrations of copper were similar in control and AAA subject groups while a highly significant decrease was observed for zinc. As zinc is also a determinant factor in the immune system, zinc-deficient subjects such as AAA patients may experience increased susceptibility to a variety of pathogens. The drop in zinc levels also resulted in a significant increase of the Cu/Zn ratio (from 1.14 ± 0.21 in controls to 1.43 ± 0.42 in patients with AAA size >50 mm). Several papers have shown that such an increase was significantly associated with lipid peroxides levels.[31,32] Even at physiological levels, copper can exhibit pro-oxidant activities by inducing free radical formation (Fenton reaction), resulting in lipid peroxidation.[33] By contrast, zinc acts as an inhibitor of free radical reaction induced by copper.

When compared with control subjects, plasma levels of lipid peroxides (–OOH) were not significantly increased in AAA patients. This contradicts Papalambros *et al.*[21] who showed that malonaldehyde levels, used as an index of lipid peroxidation, were significantly higher in AAA patients. This finding, however, have to be taken cautiously since it is well known that MDA detection by TBAR test (ThioBarbituric Acid Reagents) lacks specificity.

F_2-isoprostanes, stable isomers of prostaglandins F_{2a}, are now regarded as a reliable and specific *gold*

standard of *in vivo* lipid peroxidation. Elevated plasma and/or urine concentrations of such biomarkers have been reported in cardiovascular diseases.[34–36] Lindsay *et al.*[37] showed that isoprostanes levels were significantly increased in patients with ruptured AAA when compared with those for elective repair. In our study, urine levels of isoprostanes tended to be significantly higher but only in >50 mm group. These results need to be confirmed on larger patient groups. As far as antibodies against oxidized LDL are concerned, no correlation was found with AAA size as previously described by Lindholt *et al.*[38]

As already mentioned, infiltration of inflammatory leukocytes has been recognized to promote aneurismal remodelling of the aortic wall.[1,2] In thrombus samples obtained from 29 AAA patients during surgical repair, Houard *et al.*[4] found elevated levels of MPO into the luminal layer. Thus, augmented MPO levels should also be seen in blood, which is a well-known signal of leukocytes activation. This could not be confirmed in our study maybe due to the large dispersion of MPO values in our patient groups.

In light of the above findings, we strongly believe that monitoring biomarkers of the oxidative stress in AAA patients will enhance disease surveillance and treatment, while bringing additional evidence on the understanding of AAA. The weakening of the antioxidant defences may also suggest that an antioxidant therapy could be beneficial to AAA patients. In angiotensin II-infused apolipoprotein E-deficient mice, Gavrila *et al.*[39] have shown that vitamin E inhibits AAA formation as previously evidenced by Nakahashi *et al.*[40] in a rat model. Vitamin E also decreases aortic 8-isoprostane content (OS marker) and reduces aortic macrophage infiltration. Nonetheless, in a controlled trial, Törnwall *et al.*[41] reported that vitamin E or β-carotene supplementation did not have a preventive effect for large sized AAAs among male smokers.

Conclusions

The evidence of OS in pathological situations such as AAA remains an important challenge for scientists. Based on a large battery of biomarkers of the oxidative stress assayed for the first time in AAA patients, we showed that vitamin C, β-carotene, ubiquinone, zinc, and selenium measured in blood were negatively and significantly correlated with the size of AAA. By contrast, we evidenced a positive correlation between AAA size and the Cu/Zn ratio, a potential source of ROS formation, and isoprostanes as marker of lipid peroxidation process.

Declaration of interest

All authors have disclosed affiliations with any organization of financial interest, direct or indirect in the subject matter or materials used in the manuscript.

Acknowledgements

The authors thank Mrs A. Yasici and G. Peters for technical and secretarial assistance. This study was supported by a grant from the European integrated project 'Fighting Aneurysmal Disease' (FAD, http://www.fighthing-aneurysm*org/).

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