Oxidative Stress and Thrombotic Disorders: Study in Patients with Venous Thromboembolism

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Received: 11/12/2015 Revised: 26/12/2015 Accepted: 29/12/2015

ABSTRACT

We aimed to investigate biochemical markers and the oxidant/antioxidant balance in patients with venous thromboembolism. This study was conducted as a prospective case-control study. 70 patients with venous thromboembolism enrolled to the study and 80 healthy subjects without risk factors for venous thromboembolism were selected as control group. Venous blood samples were obtained from venous thromboembolism patients during the initial diagnosis and from the control subjects. Biochemical parameters (triglycerides and HDL-cholesterol) and oxidative stress markers (malondialdehyde, carbonyl proteins, superoxide anion expressed as reduced Nitroblue Tetrazolium, nitric oxide expressed as nitrite, reduced glutathione, vitamin C, catalase, superoxide dismutase) were assayed by biochemical methods. Plasma triglyceride was increased and HDL-cholesterol levels were decreased in venous thromboembolism patients compared to control. Malondialdehyde, carbonyl proteins levels and catalase activity were high while nitric oxide and vitamin C were low in venous thromboembolism patients than control. Reduced glutathione concentrations, superoxide anion and superoxide dismutase activity were found not significant respectively. Oxidative stress may be one of the causative factors in venous thromboembolism and probably contributes to additional disorders.

Keywords: antioxidants, free radicals, lipids, oxidants, thrombosis.

INTRODUCTION

Cardiovascular diseases (CVDs) are the leading cause of mortality worldwide. Endothelial dysfunction appears in the early stages of the pathogenesis of vascular disorders and it is closely related to the progression of severe clinical complications. Venous thromboembolism (VTE) is a common thrombotic disease that encompasses both deep vein thrombosis (DVT) and pulmonary embolism (PE). VTE is increasingly reported in Africa, where confinement to bed and surgery are responsible for 18 to 57% of cases. In Algeria, the prevalence of this disease is on the rise but there are no published data on its frequency or on the thrombogenic potential of associated risk factors.

DVT is the first and the most clinical form of VTE, a complex vascular disease associated with various etiological factors.
and results in serious morbidity and mortality. It imposes significant costs on the health care system and results in loss of functions and decreased quality of life in patients. Thrombus formation and propagation depend on the presence of abnormalities of blood flow, blood vessel wall and blood clotting components, known collectively as the Virchow triad. The processes that trigger venous thrombosis are uncertain. However, the mechanisms initiating venous thrombosis are clearly very different from those initiating arterial thrombosis. Endothelial imbalance may possibly play an important role. The vascular endothelial surface normally creates a non-thrombogenic structure. When endothelial injury occurs in association with factors such as anoxia, mechanical stress, free radicals, cytokines and thrombin, this may lead to platelet activation and coagulation.

Oxidative stress, related to an imbalance between the production of oxygen free radicals and the antioxidant defense system, has been implicated in several diseases. Indeed, many studies have shown that increased oxygen free radicals and lipid peroxidation are involved in the pathogenesis of endothelial dysfunction. Reactive oxygen species (ROS) influence many physiological processes, and cause lipid peroxidation and oxidation of some specific proteins, thus affecting many intra- and intercellular systems.

Under normal physiological conditions, the antioxidants are responsible for cellular protection against oxidative stress, namely, by the free radical scavenger enzyme superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px). Thereby maintaining the vasorelaxant and antithrombotic effects of NO in the vasculature. These scavengers are strategically compartmentalized in subcellular organelles within the cell to provide maximum protection.

Oxidative stress is an important mediator of both abnormal platelet function and dysfunctional endothelium-dependent vasodilatation in the setting of cardiovascular disease. Superoxide anion (O2−) is an important source of oxidative stress, has direct effects, and limits the biological activity of NO. Excessive vascular superoxide production has been demonstrated in hypercholesterolemia as well as other disease states associated with endothelial dysfunction. Antioxidants work together in human blood cells against toxic reactive oxygen species. Reduced glutathione plays a major role in the regulation of the intracellular redox state of vascular cells by providing reducing equivalents for many biochemical pathways.

Traditionally, VTE and atherosclerotic cardiovascular disease are viewed as two separate entities, mainly due to different clinical presentations and to different pathophysiological mechanisms. Since the publication of several studies for the past few years, a possible association between venous and arterial thrombotic disorders has been suggested.

A meta-analysis of case–control studies and cohort studies found a significant association between low HDL-cholesterol levels, high triglyceride levels and VTE.

The aim of the present work was to determine oxidative stress markers during thromboembolism disease. Therefore, several markers of oxidative stress were assessed by measuring the concentrations of plasma vitamin C, superoxide anion, nitric oxide, MDA, carbonyl proteins, and the activities of erythrocyte CAT, SOD, and reduced glutathione (GSH) in thromboembolism subjects. Changes in serum lipid levels were also determined. The present study aimed to understand how thromboembolism affects the oxidant/antioxidant status.
MATERIALS AND METHODS

Study population: The study included two populations. 80 healthy subjects, recruited among the blood donors at the regional center of blood transfusion of Tlemcen, with mean age 36.12 ± 3.85 years; and 70 thromboembolism patients, with mean age 34.18 ± 4.62 years, recruited at the cardiology department of Tlemcen Hospital, Tlemcen city (west Algeria) between November 2013 and June 2014.

These patients did not present any other pathology. For therapeutic management, we can usually use in medical practice non-fractionated heparin or low molecular weight heparin, sometimes substituted by antivitamin K therapy. The study was performed according to the Declaration of Helsinki. All participants in this study were informed about the goals and the work in progress, and were asked to give their written consent beforehand. Investigations of patients as well as blood sampling conditions were subjected to a strict code of ethics. The protocol was approved by the Tlemcen University Hospital Committee for Research on Human Subjects.

An information sheet was drawn up for each patients and witness in order to detect the risk factors in the two studied populations; namely the age, the sex, the body mass index (BMI, Kg/m^2), nicotinism, the family antecedents of thromboembolism disease, inflammatory diseases, bacterial infections and traumatism.

Blood samples: Venous blood samples were collected in heparinized tubes always in the morning, at the same time (from 08:00 A.M. to 8:30 A.M.) and after 12 hours of fasting. After centrifugation, plasma was separated for vitamin C, O_2^-, MDA, NO, PCAR and GSH determinations.

The remaining erythrocytes were washed three times with two volumes of isotonic saline solution and hemolysed by the addition of cold distilled water (1/4), stored in the refrigerator at -4°C for 15min and the cell debris was removed by centrifugation (2000g, 15min). The hemolsyates were appraised for erythrocyte oxidant / antioxidant status.

Determination of biochemical parameters: Plasma lipoprotein fractions (HDL, d<1.21 g.mL^{-1}) were separated by sequential ultracentrifugation in a Beckman ultracentrifuge (Model L5-65, 65 Ti rotor), using sodium bromide for density adjustment.

Plasma triglycerides and HDL-cholesterol contents were determined by enzymatic methods (Kits Sigma Chemical Company, St Louis, MO, USA).

Determination of markers of the oxidant/antioxidant status: NO was determined by the method of Guevara et al. [19] after plasma deproteinizing procedure (using methanol: diethyl ether; 3:1 mixture v/v). Nitrite and nitrate levels were measured together; nitrate being previously transformed to nitrite by cadmium reduction. Nitrite was assayed directly spectrophotometrically at 492 nm, using the colorimetric method of Griess (Griess reagent: 1 g/L sulfanilamide, 25 g/L phosphoric acid, 1 and 0.1 g/L N-1-naphthylethlenediamine). Calibration curves were made with sodium nitrite in concentrations from 1 to 50 μmol/ L. The determination of the superoxide anion (O_2^-) was based on Nitro Blue Tetrazolium (NBT) reduction in monofarmazan by O_2^-.

The blue formazan was dissolved using 2M potassium hydroxide and dimethylsulfoxide and its formation was monitored spectrophotometrically at 560 nm using the molar extinction coefficient (1.5x10^3 M^{-1} . cm^{-1}).

Plasma MDA was estimated by the method of Draper and Hadley et al. [21] using thiobarbituric acid (TBA). Absorbance was measured at 532 nm. The results were expressed as micromoles of MDA, using the molar extinction
coefficient of chromophore (1.56 x 10^5 M^-1 cm^-1).

Plasma carbonyl proteins, markers of protein oxidation (PCAR) were assayed by 2, 4-dinitro phenyl hydrazine reaction. [22]

Vitamin C levels were determined in plasma using dinitro phenyl hydrazine, thiourea and copper sulfate according to the method of Roe and Kuether. [23]

The CAT activity (CAT, EC 1.11.1.6), was measured by spectrophotometric analysis of the decomposition rate of hydrogen peroxide according to the method of Aebi. The reaction was initiated by additional of hemolysate to the reaction mixture containing phosphate buffer (0.05 M, pH 7.2) and H_2O_2. Change in absorbance was recorded spectrophotometrically at 240 nm. [24] Enzyme activity was expressed as U/g of hemoglobin (Hb).

The assessment of the enzymatic activity of SOD (SOD, EC 1.15.1.1) was measured by the NADPH oxidation procedure and is expressed as units of SOD/g of Hb. [25]

Hemolysate reduced glutathione (GSH) levels were assayed by a colorimetric method based on the reduction of 5, 5-dithiobis-(2-nitrobenzoic) acid (DTNB) by GSH to generate 2-nitro-5-thiobenzoic acid which has yellow color, according a Sigma Aldrich Kit (Saint Louis, MO, USA). The absorbance at 412 nm was measured, and the GSH concentration was then determined with the GSH standard curve.

**Statistical analysis:** The results obtained were expressed as means ± standard deviation (SD), and were further subjected to one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test. These calculations were performed using STATISTICA version 4.1 (STATSOFT). The significance level was set at P<0.05.

### RESULTS

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Control</th>
<th>Case</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>80</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>36.12 ± 3.85</td>
<td>34.18 ± 4.62</td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>34</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>Women</td>
<td>46</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>21.20 ± 2.52</td>
<td>23.08 ± 3.18</td>
<td></td>
</tr>
<tr>
<td>Diabetes (%)</td>
<td>/</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>HTA (%)</td>
<td>/</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>Smoking (%)</td>
<td>/</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>Alcohol (%)</td>
<td>/</td>
<td>/</td>
<td></td>
</tr>
<tr>
<td>Family antecedents heart (%)</td>
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<td>22</td>
<td></td>
</tr>
<tr>
<td>Personal antecedents heart (%)</td>
<td>2</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>PE, n</td>
<td>/</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>DVT, n</td>
<td>/</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td>DVT + PE, n</td>
<td>/</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Triglycerides (g/L)</td>
<td>0.98 ±0.35</td>
<td>1.26 ± 0.24</td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>1.62 ±0.42</td>
<td>2.03 ± 0.23</td>
<td></td>
</tr>
<tr>
<td>Women</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>HDL-C (g/L)</td>
<td>0.45 ± 0.12</td>
<td>0.21 ±0.06</td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>0.53 ± 0.14</td>
<td>0.24 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>Women</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
</tr>
</tbody>
</table>

The results are expressed as the means ± standard deviation (SD). BMI: Body mass index (weight / height²); PE: pulmonary embolism, DVT: deep vein thrombosis; HTA: hypertension arterial; HDL-C: High Density Lipoprotein-Cholesterol.

Baseline characteristics of the cases and their matched controls are shown in Table 1. The mean age of patients was 34.18 ± 4.62 years. The 70 cases were 12 isolated PE, 44 DVT of the lower limb and 14 DVT associated with PE. Median BMI was 23.08 ± 3.18 kg/m² for cases and 21.20 ± 2.52 kg/m² for controls. The HDL-Cholesterol levels were decreased in cases than in controls, whereas a significant increase in
triglycerides levels was found in VTE patients (Table 1).

**Redox balance:** Oxidant/antioxidant status alterations were marked by a significant decrease in vitamin C and plasma concentration of NO, and a significant increase in plasma concentration of MDA, plasma and erythrocyte PCAR and CAT activity in cases compared to control values (figure 1, table 2, figure 2).

The results are expressed as the means ± standard deviation (SD). GSH: reduced glutathione. Values with different superscript letters (a, b, c, d) are significantly different (P<0.05).

### Figure 1: Plasma vitamin C and GSH levels in case and control

### Table 2: Erythrocyte and Plasma oxidants levels in case and control

<table>
<thead>
<tr>
<th></th>
<th>Men</th>
<th>Women</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NO (µmol/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td>21.38 ± 3.31</td>
<td>26.17 ± 3.66</td>
<td>18.09 ± 3.20</td>
</tr>
<tr>
<td>Erythrocyte</td>
<td>34.83 ± 4.10</td>
<td>43.93 ± 5.11</td>
<td>34.80 ± 4.08</td>
</tr>
<tr>
<td>O₂⁻ (µmol/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td>32.32 ±1.18</td>
<td>39.79 ± 0.78</td>
<td>30.69 ± 0.48</td>
</tr>
<tr>
<td>Erythrocyte</td>
<td>550.95 ±1.21</td>
<td>761.2±0.524</td>
<td>579.21±1.09</td>
</tr>
<tr>
<td>PCAR (µmol/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td>6.23 ± 1.99</td>
<td>14.26 ± 2.66</td>
<td>5.33 ±0.83</td>
</tr>
</tbody>
</table>

The results are expressed as the means ± standard deviation (SD). NO: nitric oxide; O₂⁻ : superoxide anion; PCAR: carbonyl proteins; MDA: malondialdehyde. Values with different superscript letters (a,b,c,d) are significantly different (P<0.05).
The results are expressed as the means ± standard deviation (SD). SOD: superoxide dismutase. Values with different superscript letters (a, b, c, d) are significantly different (P<0.05).

The increase concentration of plasma and erythrocyte O₂⁻ and GSH was not significant. This is also the case for the decrease levels of SOD activity and erythrocyte concentration of NO which was not significant compared to control values. (Figure 1, table 2 and figure 2).

**DISCUSSION**

The previous Meta analytic results showed that the patients with a high TG and a low HDL-cholesterol were likely to suffer VTE. [18] There was correlation between VTE and metabolic syndrome, including a low-level of HDL-cholesterol. [26,27] The level of HDL-cholesterol was also lower in patients with recurrent venous embolism. [28] All these suggested that the VTE patients had a lower level of HDL. The results of the present study served to further confirm those of previous studies.

High-density lipoprotein participates in the process of reverse transport of cholesterol. It has a variety of functions, such as anti-inflammatory, antioxidation, and protection of the vascular endothelium. As demonstrated by a large body of epidemiological data, there was a negative correlation between the level of HDL-cholesterol and the occurrence of coronary heart disease. Some studies showed that the level of HDL-cholesterol was probably the manifestation of one kind of pathological state. The patients with pulmonary arterial thrombus had a higher probability of suffering coronary atherosclerosis and acute myocardial infarction. [17,29] Atherosclerosis is also a risk factor of pulmonary arterial thrombosis. The patients with atherosclerosis had a higher probability of suffering PE. Atherosclerosis and PE may share some common risk factors. [17] The formation of venous thrombosis is mainly related with the activation of clotting factors and the stasis of venous blood flow. In addition, platelets also play a partial role in the formation of venous thrombosis. The result of current research suggested that the abnormal metabolism of blood lipids, including a reduced level of HDL-cholesterol and an elevated level of TG, affected the functions of vascular endothelium and increased the activity of. [30,31] In the mean time, HDL could boost the production of eNOS in endothelial cells so as to further increase the release of nitric oxide (NO) and enhance the bioavailability of NO. [32,33] Thus, HDL could not only improve the vasodilatory function, but also inhibit the initiator of an extrinsic pathway of blood coagulation-tissue factor, [34] and suppress the activity of clotting factors, Va and VIIIa. [35] Our results are in agreement with what has been said in the literature.

We investigated the status of lipid peroxidation and oxidative damage in VTE patients and found that MDA and PCAR levels were significantly elevated in these patients compared with controls.

These increased levels of MDA indicate lipid peroxidation in patients with DVT. The only study we could find in the literature on this subject is the study of Re et al. [36] They found higher MDA and hydroxynonenal (HNE) and myeloperoxidase (MPO) levels in DVT patients compared to the healthy controls.

Increased blood MDA levels are possible consequent of oxygen free radical-mediated damage of the membrane lipid while the increase in the blood PCAR level may be related to the oxidative damage of protein by elevated oxygen free radicals in the VTE patients. Carbonylation of proteins is an irreversible oxidative damage, often leading to the loss of protein function, which is considered a widespread indicator of severe oxidative...
damage and disease-derived protein dysfunction. \[37,38\]

NO is synthesized in the endothelial cells from the conversion of L-arginine to L-citrulline through the tightly regulated activity of endogenous nitric oxide synthase (NOS). \[39\]

It modulates vasomotor tone, inhibits platelet aggregation, and smooth muscle cell proliferation. Oxidative stress decreases the half-life of NO. NO may play a role in haemostatic plug formation and in thrombotic process with the effects on blood platelets. However, in vivo studies on the role of NO in thrombotic process are not well defined.

Under normal physiological conditions, the antioxidants are responsible for cellular protection against oxidative stress, namely, by the free radical scavenger enzyme SOD, CAT, and glutathione peroxidase (GSH-Px), \[40\] thereby maintaining the vasorelaxant and antithrombotic effects of NO in the vasculature. \[13\] These scavengers are strategically compartmentalized in subcellular organelles within the cell to provide maximum protection.

Cellular glutathione peroxidase (cGPx) is tightly coupled to the hexose monophosphate shunt through reduced nicotinamide adenine dinucleotide phosphate (NADPH), which maintains the obligate cosubstrate of cGPx, GSH and re-establishes the platelet thiol redox state via glutathione reductase. Glutathione depletion in platelets leads to attenuated cGPx activity and increased lipid peroxidation. \[41\] Increased lipid peroxides, in turn, lead to an increased likelihood of lipid peroxyl radical formation (LOO), which can react with and inactivate NO by forming lipid peroxynitrites (LOONO). Cellular GPx potentiates the inhibition of platelet function by NO by reducing both LOOH and derivative LOONOs.

Experimental evidence suggests that antioxidant status is important in normal platelet function and the prevention of thrombosis. Studying cyclic flow variations in rabbit carotid arteries, Meng et al. showed that platelet-mediated thrombosis can be attenuated by the intravenous infusion of SOD. \[42\] Consistent with these observations, in a model of endothelium-injured canine coronary arteries, Ikeda et al. demonstrated that cyclic flow variation was attenuated by intravenous infusion of SOD and CAT. \[43\] Infusion of xanthine and hypoxanthine or hydrogen peroxide, however, significantly increased cyclical flow variation. These data suggest that reactive oxygen species contribute to platelet activation and thrombosis. Some authors suggest that decreased antioxidant enzymes are associated with retinal vein and central vein thrombosis and that increased oxidative stress may predispose to venous occlusive disorders or venous thrombosis. \[44,13\]

The detrimental effect of ROS is expected to be uniquely most profound in the lung due to its constant exposure to the high oxygen tension of the ambient atmosphere. Consequently, the lung has evolved significant defense mechanisms against cellular damage from reactive oxygen species, which most notably include the family of SOD, the peroxidase CAT, and GSH. \[45\] Superoxide dismutase has three isoforms and catalyzes the breakdown of superoxide anion to hydrogen peroxide, and catalase accelerates the breakdown of hydrogen peroxide to water. GSH is a tripeptide containing the amino acid cysteine, and with its sulphydryl group, acts as one of the major antioxidants present in the lung.

Finally, we found that VTE patients had lower levels of vitamin C than controls. The antioxidant properties of vitamin C are thought to act synergistically with those of vitamin E, decreasing the formation of peroxyl radicals and blocking lipid peroxidation. Vitamin C also has an action on endothelial vasodilator function in heart failure by increasing available NO,
but high doses of vitamin C have also been associated with decreased levels of NO production by endothelial cells. One clinical trial reported that vitamin C slowed progression of atherosclerosis in men and women over 55 years of age. The elderly and other groups in the population, including males, smokers, diabetics and hypertensive, who are at increased risk of cardio heart disease, have been found to have lower-than-average vitamin C blood levels. Women taking oral estrogen contraceptives may also have below-average vitamin C levels.

CONCLUSION
Our data showed that patients with DVT have increased oxidative stress compared with the healthy volunteers. Meanwhile, previous studies have demonstrated a negative correlation between the level of HDL-cholesterol and the occurrence of coronary heart disease. The present study also revealed that the patients with pulmonary arterial trunk embolism had a higher tendency of death, and the HDL-cholesterol levels tended to be lower than it is in the survivor. Further studies are needed to confirm whether the elevation of HDL-cholesterol is able to prevent a second venous thrombosis in VTE patients and whether the elevation of HDL-cholesterol is able to improve prognosis in PE patients. We may have only observed a vague phenomenon between HDL-cholesterol and pulmonary embolism, but we think more studies are needed to reveal a substantial relationship.

ACKNOWLEDGEMENTS
This work was supported with a financial support of the Algerian Health Investigation Office (ANDRS). Our thanks go to all volunteers.

Conflict of interests: The authors declare that there is no conflict of interests regarding the publication of this paper.

REFERENCES


