Evaluation of Oxidative DNA Damage and Thiol–Disulfide Homeostasis in Patients with Aortic Valve Sclerosis

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ABSTRACT
Objective: The free radicals in the organism are important events in aortic valve sclerosis (AVS) because they cause conditions such as cell proliferation, growth arrest, and/or apoptosis and oxidation of low-density lipoprotein (LDL). This study was to evaluate DNA damage in patients with AVS and its relationship with thiol–disulfide homeostasis.

Methods: Forty AVS subjects (30 female) were enrolled in this study and compared with control group. The diagnosis of AVS was made by comprehensive echocardiography. Biochemical parameters were measured in the sera of the control and AVS subjects.

Results: In the AVS group, total oxidant status (TOS), total antioxidant status (TAS), oxidative stress index (OSI), disulfide, total thiol, natural thiol, disulfide/total thiol, disulfide/natural thiol, and oxidative 8-OH deoxyguanosine levels were significantly lower than those of the control group, whereas natural thiol/total thiol levels were significantly found to be higher. In addition, there was a statistically negative correlation between OSI and TAS in patient and control groups, and a positive correlation between OSI and TOS and natural and total thiol parameters. The results show DNA damage and impaired thiol-disulfide homeostasis.

Conclusion: Our findings suggest that increased oxidant stress may signify an important point in the onset and progression of AVS. Therefore, adding antioxidants to treatment may shed light on new therapeutic targets for reinforcing the antioxidant system, slowing or even stopping aortic valve stenosis.

Keywords: Reactive oxygen species, oxidative stress, oxidative DNA damage, free radicals, antioxidants, thiol-disulfide homeostasis

INTRODUCTION
Aortic valve sclerosis (AVS) is defined as thickening and calcification of aortic valve patients in cases where ventricular outflow is not obstructed.1–3 AVS is the most common valve disease in developed countries and is also common in western countries. It is found in about 25% of people in the 65 age group and increases to 50% in the 80 age group. Recent studies show that aortic valve disease is common in the US adult population and causes more than 28,000 deaths and 48,000 hospitalizations per year.4,5 Most studies have proved that patients with AVS have an augmented incidence of cardiovascular events and mortality.6,7 AVS developing approximately takes six decades. Then, it takes around one decade for a patient to progress aortic valve stenosis. There is also no randomized controlled trial on drug therapy in aortic sclerosis. Therefore, no medical interventions are able to delay or stop the AVS progression.8

Reactive nitrogen species (RNS), reactive oxygen species (ROS), and other radicals are produced as a normal result of metabolism.9–11 When ROS and RNS are overproduced, they cause oxidative and nitrosative stress, respectively.12–14 In many studies, the formation of overactive ROS has been reported to cause cellular damage and atherogenesis. In addition to the deleterious effects of ROSs, they are also expressed to be involved in various cell processes involving initiation of cell proliferation and gene expression, growth arrest, hypertrophy, and induction of apoptosis.15–18 However, research has suggested that ROS/RNS can have some detrimental effects on DNA and, indeed, trigger chromosomal aberrations, DNA strand breaks, and, consequently, DNA damage from endogenous free radical attacks, contributing to many diseases.19–22 Some studies state that an important feature of atherosclerotic plaques (APs) is oxidative DNA damage.17,23


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Thiols, which play in redox homeostasis and are composed of functional sulfhydryl groups, are important molecules of the oxidant/antioxidation system. In living systems, the thiol representing the reduced state and the disulfide groups representing the oxidized state are regularly converted into each other to maintain stability between the thiol and disulfide groups. Dynamic thiol/disulfide balance is defined as a new oxidative stress (OS) marker, and it has been stated in studies that it plays a role in the pathophysiology of a lot of diseases, including diabetes and cardiovascular disorders.

To our best knowledge, there are no studies researching thiol-disulfide homeostasis in patients with AVS. Therefore, we aimed to evaluate serum thiol/disulfide status, total oxidant status (TOS), total antioxidant status (TAS), and the levels of 8-OH deoxyguanosine (8-OHdG) in these patients and search the role of active stress pathways in the pathogenesis of AVS and whether the results obtained correlated with disease severity.

**METHODS**

**Study Population**

All subjects were included in the study after obtaining their informed consent. A total of 80 subjects, 40 patients (30 females and 10 males) with AVS and 40 controls of healthy volunteers, were included in this study. The diagnosis in these patients was made by a cardiologist based on echocardiography scans. Echocardiographic evaluation was performed to determine whether the control group had heart pathology. The subjects in the control group had normal aortic valve tips and did not have any cardiovascular disorders or other diseases (such as liver, kidney disease, diabetes, hypertension, and cancers). Pregnant patients and individuals with severe diseases (such as renal dysfunction, lung disease, blood diseases, rheumatoid arthritis patients, cancer, liver disease, congenital heart disease, and other heart diseases) were not included in the study to prevent possible effects on serum biochemical parameters. This study was approved by Gaziantep University Clinical Research Ethics Committee with a protocol no. 2018/195 on November 7, 2018.

**Echocardiography**

The standard for diagnosing AVS was dependent on the hemodynamic and morphologic findings in the echocardiographic study in the aortic valve by the wall thickness of a given hump (2-6 mm at minimum one abnormal leaflet per valve), with a transaortic flow rate of <2.5 m s⁻¹.

**Biochemical Analysis**

TOS, TAS, thiol-disulfide levels, and the 8-OHdG, the marker of DNA damage, from the venous blood samples taken from the control and patient groups were measured in xxx University Medical Faculty Hospital medical biochemistry research laboratory. To obtain serum, blood samples were centrifuged at 3,500 rpm for 8 minutes. Serum samples obtained were placed into ependrof tubes and stored at −80°C until analysis.

**Total Oxidant Status Assay**

TOS was determined as described by Erel. TAS measurement was performed using an automated analyzer (Beckman Coulter AU480 Chemistry Analyzer) fully automatic TOS kit (Rel Assay DC, Gaziantep, Turkey). Results are expressed in μmol H₂O₂ equiv. L⁻¹.

**Total Antioxidant Status Assay**

TAS was determined as described by Erel. TAS measurement was performed using a fully automatic RAS kit in the Beckman Coulter AU480 Chemistry autoanalyzer (Rel Assay DC, Gaziantep, Turkey). Results are expressed in mmol Trolox equiv. L⁻¹. Oxidative stress index (OSI) was calculated by using TOS and TAS values. First, the TAS unit was turned into μmol L⁻¹. Then, TOS values were divided by TAS values and multiplied by 100. The resulting ratio was expressed as OSI.

**Serum Thiol-Disulfide Measurement**

In this method, disulfide, total thiol, and native thiol concentrations were detected. Then, other parameters were calculated, which expressed as μmol L⁻¹.

**Serum 8-OH Deoxyguanosine Measurement**

8-OHdG levels were determined by ELISA method, which expressed as ng mL⁻¹.

**Statistical Analyses**

Data analysis and receiver operating characteristic (ROC) curve analysis were performed using Statistical Package for Social Sciences for Windows, version 11.5 (SPSS Inc.; Chicago, IL, USA). The Shapiro–Wilk test was used to determine whether continuous variables were normally distributed. Results obtained were expressed as mean ± SD. Differences between the groups in the normally distributed variables were analyzed using independent sample’s T test. The Mann–Whitney U test was used to analyze the differences between the non-normal distribution data. In the correlation analysis, Pearson correlation analysis was used for normal variables and Spearman correlation analysis for non-normal distribution. P values <.05 were considered statistically significant.

**RESULTS**

Samples were run duplicate, and their means were used for statistical evaluations. TAS, TAS, OSI, thiol/disulfide parameters, and 8-OHdG, which are indicators of oxidative DNA damage, levels were measured in the sera of patients in AVS and control groups (Tables 1-3 and Figure 1). In the AVS group, TAS, OSI, and 8-OHdG levels were found to be significantly lower when compared with the control group. However,
native thiol/total thiol levels were found to be statistically higher than the control group. ROC analysis was used to determine the predictive threshold of serum TOS, TAS, OSI, and 8-OHdG levels (Figure 2).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control Group (n: 40) (Mean ± SD)</th>
<th>Patient Group (n: 40) (Mean ± SD)</th>
<th>P Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native thiol (μmol L⁻¹)</td>
<td>300.19 ± 18.12</td>
<td>289.72 ± 21.64</td>
<td>.05</td>
</tr>
<tr>
<td>Total thiol (μmol L⁻¹)</td>
<td>323.79 ± 18.27</td>
<td>309.96 ± 24.87</td>
<td>.01</td>
</tr>
<tr>
<td>Disulfide (μmol L⁻¹)</td>
<td>11.80 ± 1.86</td>
<td>10.12 ± 3.54</td>
<td>.01</td>
</tr>
<tr>
<td>Disulfide/native thiol (%)</td>
<td>3.94 ± 0.68</td>
<td>3.48 ± 1.17</td>
<td>.05</td>
</tr>
<tr>
<td>Disulfide/total thiol (%)</td>
<td>3.65 ± 0.58</td>
<td>3.23 ± 1.01</td>
<td>.05</td>
</tr>
<tr>
<td>Native thiol/total thiol (%)</td>
<td>92.69 ± 1.16</td>
<td>93.52 ± 2.03</td>
<td>.05</td>
</tr>
<tr>
<td>Total antioxidant status (mmol Trolox equiv. L⁻¹)</td>
<td>1.58 ± 0.2</td>
<td>1.49 ± 0.17</td>
<td>.05</td>
</tr>
<tr>
<td>Total oxidant status (μmol H₂O₂ equiv. L⁻¹)</td>
<td>14.35 ± 0.59</td>
<td>4.77 ± 0.56</td>
<td>.01</td>
</tr>
<tr>
<td>Oxidative stress index (arbitrary unit)</td>
<td>0.24 ± 0.05</td>
<td>0.31 ± 0.06</td>
<td>.01</td>
</tr>
</tbody>
</table>

The results obtained in mean and standard deviation values were compared between the two groups. P < .05 was considered statistically significant.

In the correlation analysis, there was a statistically significant negative correlation between TAS and OSI in patient and control groups and positive correlations between TOS and OSI and total and native thiol parameters (Figures 3 and 4).
The mean descriptive data of the patient and control groups are given in Table 4. Body mass index (BMI) in females in the AVS patient group was significantly higher than the control group ($P = 0.003$), whereas there was no difference in men. High blood pressures were detected in both genders in the AVS patient group compared with the healthy control group ($P < 0.05$). In addition, while 42% of the AVS patient group had diabetes, none of the control group had diabetes. In addition, there was no significant difference in diabetes prevalence between different genders in the AVS patient group ($P > 0.05$) (see more detail in Table 4). The ages of the individuals in the control group ranged from 42 to 65 years, while the age of the individuals in the AVS group was between 39 and 65. In addition, BMI was found to be higher in the patient group compared with the control group (see more detail in Table 5).

**DISCUSSION**

With increasing evidence of a direct role of OS-induced DNA damage in the experimental model of atherosclerosis, a direct mechanism has been proposed to define the role of DNA
Table 4. Descriptive Analyses of the Study Groups

<table>
<thead>
<tr>
<th></th>
<th>Gender</th>
<th>Patient (Mean ± SD)</th>
<th>Control (Mean ± SD)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>Female</td>
<td>56.6 ± 7.6</td>
<td>57.4 ± 7.0</td>
<td>.676</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>56.8 ± 6.2</td>
<td>53.4 ± 7.5</td>
<td>.287</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>56.6 ± 7.2</td>
<td>56.4 ± 7.3</td>
<td>.878</td>
</tr>
<tr>
<td>BMI</td>
<td>Female</td>
<td>34.3 ± 6.5</td>
<td>29.4 ± 5.8</td>
<td>.003</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>28.7 ± 2.9</td>
<td>28.7 ± 3.4</td>
<td>.992</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>32.9 ± 6.3</td>
<td>29.2 ± 5.3</td>
<td>.006</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>Female</td>
<td>145.1 ± 15.9</td>
<td>121.7 ± 9.0</td>
<td>.000</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>136.7 ± 15.3</td>
<td>120.0 ± 8.2</td>
<td>.007</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>142.9 ± 16.0</td>
<td>121.3 ± 8.7</td>
<td>.000</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>Female</td>
<td>74.5 ± 11.7</td>
<td>64.7 ± 7.8</td>
<td>.000</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>72.3 ± 9.6</td>
<td>63.8 ± 5.9</td>
<td>.028</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>74.0 ± 11.1</td>
<td>64.5 ± 7.3</td>
<td>.000</td>
</tr>
</tbody>
</table>

BMI, body mass index.

Table 5. Descriptive Analyses of the Study Groups

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Minimum</th>
<th>Maximum</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>80</td>
<td>39.0</td>
<td>65.0</td>
<td>56.6</td>
<td>7.2</td>
</tr>
<tr>
<td>BMI (kg m$^{-2}$)</td>
<td>80</td>
<td>21.1</td>
<td>54.6</td>
<td>31.1</td>
<td>6.0</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>80</td>
<td>108.0</td>
<td>168.0</td>
<td>132.13</td>
<td>16.8</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>80</td>
<td>54.0</td>
<td>99.0</td>
<td>69.2</td>
<td>10.5</td>
</tr>
</tbody>
</table>
damage in the development of cardiovascular diseases. It is now known that aortic valve stenosis is the final stage of a disease that develops from microscopic early alterations to aortic sclerosis and then to severe bio-mineralization in a subset of patients. In order to influence the progression of the transition from sclerosis to stenosis, it is necessary to know the earliest stages of the disease, so that the effects of directed therapy on the microscopic processes in the valve patients can be measured. Despite best efforts, progress in understanding, diagnosing, and treating calcific aortic valve disease has prevented in vivo measurement of dynamic molecular events associated with early calcific changes in the valves. In spite of the high prevalence of aortic sclerosis, little is known about its developmental stages and pathogenetic mechanisms. The present study provides several new ideas about pathogenesis and progression of AVS.

First, increased oxidant stress and impaired thiol-disulfide homeostasis in AVS patients may cause increased production of ROS as the result of the reduced antioxidant defense system, causing DNA damage (Table 1 and Figures 1-4). However, the oxidative DNA damage caused by the free radical attack in these patients remains a weakly studied area. Free radicals are produced continuously as a result of normal metabolism. In living organisms, excessive ROS/RNS production and inadequate and/or disruption of the enzymatic and nonenzymatic antioxidant defense system that neutralizes the free radicals formed cause oxidative and nitrosative stress, respectively. Since OS and one of its consequences, DNA damage, play a vital role in the pathogenesis of many illnesses, it is important to understand this and to clarify how improvements are needed in this area.

In contrast, low/medium concentrations oxidative/nitrosative of ROS/RNS have beneficial effects on living organisms. They include physiological roles in cellular responses to noxia, as, for example, in defence against infectious agents, in the function of many cellular signaling pathways, and the induction of a mitogenic response. ROS/RNT overproduction causes significant injury to cell structures including membranes and lipids, DNA, and proteins.

Indeed, studies have suggested that ROS/RNS can trigger chromosomal aberrations extensive, DNA strand breaks, and DNA damage, and that important damage to DNA from endogenous free radical attacks contributes to cancer pathology and various neurodegenerative diseases. In our study, increased DNA damage was detected in the serum of patients with AVS. This finding supports the assumption that oxidative tissue damage is exacerbated during the formation of stenosis. 8-OHdG is an important DNA damage marker that occurs in the presence of oxidative/nitrosative stress in mammalian DNA.

Thiol groups, which have an important share in the antioxidant activity of the blood, and other antioxidants play an important role in preventing oxidative damage to biomolecules. Reactions resulting in thiol-disulfide exchange have important roles in biology. It has long been thought that these reactions have only a protein stabilizing structural purpose, but it is now clear that many enzymes are also responsible for the various dynamic functional properties. The oxidized state as disulfide groups and reduced state as thiol are regularly converted into one another as a result of normal metabolism, maintaining stability between the thiol and disulfide groups. The dynamic thiol/disulfide balance has been identified as a novel OS marker and has been shown to participate in antioxidant protection, detoxification, and apoptosis. In this study, we demonstrated that thiol/disulfide homeostasis varies against to thiol concentrations in patients with AVS. There was a statistically important decrease in the other thiol and disulfide parameters in the patient group except for the native thiol/total thiol ratio. This causes a significant decrease in the antioxidant defense system and an augment in oxidant stress.

Reduction in TAS and increased OSI and TOS in the patient group is an important evidence of the presence of OS. In addition, a statistically significant negative correlation between OSI and TAS and a positive correlation between TOS and OSI support our hypothesis. TAS measures the cleaning capacity of free radicals of the extracellular antioxidant system consisting of sulphydryl groups (mostly albumin), phenol compounds, vitamins A, C, and E, and proteins. TAS is an important reflection of the residual antioxidant status after cleaning of ROS/RNS. DNA damage is associated with OS. Therefore, this suggests that DNA damage may be due to insufficient antioxidant capacity and excessive ROS/RNS formation that contribute to the pathogenesis of the disease in patients with AVS. For this reason, the finding supports the hypothesis of OS involvement in the disease process.

Demirdağ et al., in a study in which they measured both TAS and TOS levels in plasma and human APs, found that TAS and TOS levels in plasma were significantly increased compared to APs. They showed that the severity of atherosclerosis was significantly related to plasma antioxidant levels rather than tissue levels. They suggested that the improvement of plasma TAS may represent an important target for the treatment of atherosclerosis disease. In another study by Klimiuk et al., it is emphasized that enzymatic and nonenzymatic antioxidant defense system defects in patients with chronic heart failure, and oxidative damage occurs in proteins and lipids in plasma/erythrocytes. They note that redox homeostasis disorders often worsen with the progression of heart failure, and some parameters of OS in saliva can be used as potential diagnostic biomarkers.

Limitations
The most important limitations are the limited number of patients, and the fact that it is performed as a single center study. A multicenter experiment will more accurately reflect real-world data. Finally, a prospective follow-up of these patients may show different rates of progression to clinical aortic stenosis among patients with normal and low levels of these parameters.

CONCLUSION
This is the first research to detect impaired thiol/disulfide homeostasis in patients with AVS. We have also detected augmented OS and DNA damage and decreased antioxidant capacity in these patients. Our findings suggest that increased
oxidant stress might represent a significant point in the onset and progression of AVS. Therefore, adding antioxidants to the treatment may shed light on new therapeutic targets for the recovery of thiol disulfide balance, slowing or even stopping AVS. However, more extensive further studies on the subject were needed.

Ethics Committee Approval: Ethical committee approval was received from the Gaziantep University Clinical Research Ethics Committee (protocol no. 2018/195 on November 7, 2018).

Informed Consent: Written informed consent was obtained from all participants who participated in this study.

Peer-review: Externally peer-reviewed.


Conflict of Interest: The authors have no conflicts of interest to declare.

Financial Disclosure: The authors declared that this study has received no financial support.

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