Oxidative stress in relation to lipid profiles in different stages of breast cancer
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The changes in the levels of MDA, nitrite, vit. E, lipids (total cholesterol and triglycerides) and lipoproteins (HDL and LDL cholesterol) were estimated among breast cancer patients, in relation to different clinical stages (stage I to IV). MDA and nitrite levels were increased in breast cancer patients, irrespective of clinical stage, as compared to controls (p<0.01). Their levels were also significantly elevated from stage III to stage IV (p<0.05). In contrast, vit. E levels were decreased in all stages, as compared to control group (p<0.05), the decrease was more pronounced in stage II and IV. Compared to controls, serum triglycerides were elevated in all patient groups (p<0.05); the maximum increase was in stage IV. HDL-cholesterol decreased in all stages, when compared with control group (p<0.05). These findings support the hypothesis that reactive oxygen and nitrogen species are increased in breast cancer, especially metastases and may cause consumption of vit. E.

Keywords: Malondialdehyde; vitamin E; nitrite; cholesterol; high-density lipoprotein; low-density lipoprotein; triglycerides; breast cancer, oxidative stress

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Oxidative stress plays an important role in the pathogenesis of numerous degenerative or chronic diseases, such as cancer and atherosclerosis. In these pathological states, the increased production and/or ineffective scavenging of oxidants may play a crucial role in determining tissue injury. The levels of oxidant molecules are controlled by various cellular defence mechanisms, consisting of enzymatic (catalase, glutathione peroxidase, superoxide dismutase) and non-enzymatic (vit. E, vit. C, glutathione) components. Oxidants include reactive oxygen species (ROS), reactive nitrogen species (RNS), and sulphur-centered radicals etc. ROS are capable of altering all major classes of biomolecules, such as lipids, nucleic acids, proteins, with changes in their structure and function. Prime targets of ROS are the polyunsaturated fatty acids in cell membranes and their interaction results in lipid peroxidation. ROS-induced lipid peroxidation has been implicated in neoplastic transformation.

Nitric oxide (NO) which is synthesized by nitric oxide synthase is a polyfunctional signaling molecule controlling processes of vasodilatation, platelet aggregation, immunocytotoxicity and carcinogenesis. Analysis of nitrite and nitrate has been used extensively as an index of endogenous production of NO in the biological systems, with respect to various pathological processes. Reactive oxygen and nitrogen species, such as the hydroxyl radical, NO and nitrogen dioxide have been proposed to contribute to multi-stage carcinogenesis via DNA or tissue damage, mutations and chromosomal aberrations in inflamed tissues. Excess NO can damage DNA through various mechanisms. It also reacts with superoxide radical to form peroxynitrite, which is considered a major agent responsible for tissue injury.

Breast cancer is one of the most common cancers in women of developed and developing countries. Free radicals have been implicated in the pathogenesis of breast cancer. There are conflicting reports of tissue concentrations of malondialdehyde (MDA), nitrite and vit. E in breast cancer patients and only a few studies on their blood concentrations have been reported. Previously, enhanced lipid peroxidation and impairment in antioxidant defence mechanisms were demonstrated in patients with lung and breast cancers, irrespective of clinical stages. The present study was aimed to determine the extent of oxidative stress (by measuring MDA, nitrite and vit. E, lipids and lipoproteins in the serum), and to evaluate the relationship between oxidative stress and different clinical stages in breast carcinoma patients.
Materials and Methods

Materials

1,1,3,3-Tetraethoxypropane (TEP), butylated hydroxytoluene (BHT), o-phosphoric acid, 2,4,6-tripyridyl-s-triazine, sulfanilamide and N-(1-naphthyl)etylenediamine were obtained from Sigma (St. Louis, MO, USA). Xylene, absolute ethanol, n-butanol, HPLC-grade meth anol, 2-thiobarbituric acid (TBA), potassium dihydrogenphosphate and dinatrium hydrogenphosphate were purchased from Merck (Darmstadt, Germany). All other chemicals used were of analytical grade.

Patients

We selected 57 female breast cancer patients admitted to Ankara Oncology Educational and Research Hospital, Ankara, Turkey. Patients did not receive any chemo or radiotherapy before blood collection. None of the patients were using oral contraceptives, hormones, or vitamins and all were non-smokers. In order to eliminate confounding variables, patients with concomitant diseases such as diabetes mellitus, liver disorders or rheumatoid arthritis were excluded. All patients studied received similar diet. Age of patients ranged between 29 and 92 yr (mean 52.1 yr). The clinical staging was done according to the TNM-UICC classification: 2 (3.5%) patients were classified as stage I, 17 (29.8%) as stage II, 20 (35.1%) as stage III, and 18 (31.6%) as stage IV. Twenty-four women (aged 24-70 yr, mean 47.3 yr), who were admitted to the hospital with minor surgical problems, other than breast diseases or any other malignancies, served as controls.

Fasting blood samples (approx. 10 mL) were collected from patients and controls under aseptic conditions by venipuncture. The blood samples were centrifuged at 3000 rpm for 10 min at 4ºC. The sera of patients and controls were stored at -70 ºC until their analysis.

The study was approved by the ethical committee of the hospital. Patients and controls were informed of the study, and their consents were taken during blood collection.

MDA in serum

The MDA concentration in serum was measured by high performance liquid chromatography (HPLC)\textsuperscript{25}. The chromatographic system consisted of a Hewlett-Packard Chromatographic Series 1050 and gradient pump and Hewlett-Packard model 1046A programmable fluorescence detector. The column was a Hewlett-Packard Lichrospher RP 18-5 250 x 4.6 mm. Mobile phase comprised of methanol-phosphate buffer at pH 6.8 (40:60, v/v). Pump flow-rate was 1.0 mL/min. To 50 µL serum or TEP standard, 50 µL BHT solution, 400 µL H\textsubscript{3}PO\textsubscript{4} solution and 100 µL TBA solution were added. Following heat derivatization, 250 µL n-butanol was added to each vial for extraction of the MDA-TBA complex. Upper layer was used for HPLC analysis.

Vit. E in serum

Serum vit. E was measured as described\textsuperscript{26}. To 500 µL absolute ethanol, 500 µL serum and 500 µL xylene were added. The tube was centrifuged for 5 min at 3000 rpm. The 250 µL of xylene layer was pipetted into a cuvette. To the cuvette, 250 µL of 2,4,6-tripyridyl-s-triazine (TPTZ) solution was added. Absorbance of the sample was measured at 460 nm after 4 min. Then, 50 µL ferric chloride solution was added and absorbance of the sample measured at 600 nm after 12 min.

Nitrite in serum

Nitrite was measured using Griess reagents as previously described\textsuperscript{27}. The 100 µL of serum samples were used. After adding 50 µL 1% sulfanilamide (Griess reagent 1), followed by the addition of 50 µL 0.1% N-(1-napthyl)ethylenediamine (Griess reagent 2), the absorbance was measured at 550 nm after 15 min incubation.

Lipid and lipoprotein in serum

Serum total cholesterol (TC) was measured enzymatically on the Technicon Daax 48 autoanalyzer by using a commercial kit (Beckman, Ireland). Serum triglyceride (TG) was estimated by enzymatic method (Beckman kit) on autoanalyzer. HDL-cholesterol (HDL-C) was determined by precipitating very low-density lipoproteins (VLDL-C) and LDL-C (Beckman kit). HDL-C remaining in the supernatant was determined enzymatically on the autoanalyzer as described for TC. Concentration of serum LDL-C was calculated by the standard Friedewald formula.

Statistical analysis

Kruskal-Wallis one-way analysis of variance (ANOVA) and unpaired Student’s t-test were applied to determine the significance of biochemical parameters among the clinical stages (II, III, and IV) and healthy controls, between total patients and controls. P value of <0.05 was considered as significant. Pearson correlation coefficients were
calculated for relationship between measured parameters. Data were analyzed using the statistical package program SPSS 10.0.

Results

Table 1 shows the characteristics of breast cancer patients and corresponding controls. The status of biochemical parameters studied in breast cancer patients with different clinical stages as compared to the controls is given in Table 2. Because of the small number of patients in stage I, this group didn’t statistically compare with the other groups.

Serum MDA levels were significantly elevated in stage II (p<0.01), III (p<0.01) and IV groups (p<0.01), as compared to controls. According to disease severity, significant increase in MDA levels was also observed in stage IV, when compared with stage III (p<0.05). Like MDA, serum nitrite levels were also significantly elevated in stage IV (p<0.05), when compared with control and stage III group. Vit. E levels were significantly decreased in stage II (p<0.01), III (p<0.01) and IV (p<0.01), as compared to controls; however, from stage III to stage IV, the decrease was not significant.

No significant changes were observed in serum total cholesterol and LDL-C levels in stage II, III, IV patients, as compared to controls (p>0.05). However, serum HDL-C concentration was significantly decreased in stage II (p<0.05), III (p<0.05) and IV (p<0.05), when compared with controls. Serum TG level was significantly elevated in stage IV (p<0.05), with respect to control group. Using bivariate correlation analysis of the measured parameters, a positive correlation was found between serum nitrite and MDA levels (r=0.251, p<0.05) and a negative correlation with vit. E levels (r=-0.239, p<0.05) in the whole study population. Vit. E levels were also correlated in a positive manner with HDL-C levels (r=0.310, p<0.01) and in a negative manner with MDA (r=-0.372, p<0.01) (Table 3).

Discussion

Oxidative stress caused by increased free radical generation and/or a decreased antioxidant level in the target cells and tissues is suggested to play an important role in carcinogenesis. Potentially cytotoxic agents that are important in the etiopathogenesis of several diseases are generated by the interaction of ROS and RNS. Increased oxidative stress and lipid peroxidation are implicated at various stages of carcinogenic processes. Earlier studies showed the involvement of ROS and RNS in cancer initiation and promotion.

Presence of NO is well-known in human biological fluids, suggesting its role in physiological and pathological processes. It is readily oxidized to nitrite and nitrate in biological systems. It exhibits a

Table 1— General characteristics of breast cancer patients and healthy controls

<table>
<thead>
<tr>
<th>General characteristics</th>
<th>Breast cancer patients</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of subjects</td>
<td>57</td>
<td>24</td>
</tr>
<tr>
<td>Age (years)</td>
<td>52.10±5.34</td>
<td>47.30±7.20</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.25±1.66</td>
<td>25.52±1.43</td>
</tr>
<tr>
<td>Menopausal status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Premenopausal</td>
<td>23</td>
<td>15</td>
</tr>
<tr>
<td>Postmenopausal</td>
<td>34</td>
<td>9</td>
</tr>
<tr>
<td>Clinical stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage I</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>Stage II</td>
<td>17</td>
<td>-</td>
</tr>
<tr>
<td>Stage III</td>
<td>20</td>
<td>-</td>
</tr>
<tr>
<td>Stage IV</td>
<td>18</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2—Status of MDA, nitrite, vit. E, plasma lipids and lipoproteins in different clinical stages of breast cancer patients and controls

<table>
<thead>
<tr>
<th>Category of patients</th>
<th>MDA (µmol/L)</th>
<th>Nitrite (µmol/L)</th>
<th>Vit. E (mg/dL)</th>
<th>Total -C (mg/dL)</th>
<th>TG (mg/dL)</th>
<th>HDL-C (mg/dL)</th>
<th>LDL-C (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (n=24)</td>
<td>2.27±0.21</td>
<td>5.58±1.08</td>
<td>0.88±5.80</td>
<td>176.62±7.85</td>
<td>103.37±10.74</td>
<td>59.21±2.07</td>
<td>96.87±6.34</td>
</tr>
<tr>
<td>Stage I (n=2)</td>
<td>2.07±0.79</td>
<td>7.17±0.47</td>
<td>0.89±4.50</td>
<td>186.00±1.00</td>
<td>96.00±6.00</td>
<td>63.50±2.50</td>
<td>103.50±4.50</td>
</tr>
<tr>
<td>Stage II (n=17)</td>
<td>3.96±0.28a</td>
<td>14.70±3.93</td>
<td>0.46±4.66b</td>
<td>179.41±7.21</td>
<td>132.59±13.13</td>
<td>48.11±3.54b</td>
<td>104.94±5.60</td>
</tr>
<tr>
<td>Stage III (n=20)</td>
<td>3.59±0.36a</td>
<td>10.56±2.82</td>
<td>0.55±5.41b</td>
<td>175.10±7.34</td>
<td>150.05±27.61</td>
<td>47.00±2.91b</td>
<td>99.50±6.80</td>
</tr>
<tr>
<td>Stage IV (n=18)</td>
<td>5.06±0.55c</td>
<td>21.51±5.21c</td>
<td>0.49±6.54b</td>
<td>198.06±11.18</td>
<td>169.17±29.91</td>
<td>51.50±2.78b</td>
<td>116.89±7.17</td>
</tr>
</tbody>
</table>

As compared to controls, a p<0.01, b p<0.05; as compared to stage III, c p<0.05.

Table 3—Pearson correlation coefficients between measured parameters in the total group

<table>
<thead>
<tr>
<th></th>
<th>Nitrite</th>
<th>MDA</th>
<th>Vit. E</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>p</td>
<td>r</td>
</tr>
<tr>
<td>Nitrite</td>
<td>-</td>
<td>0.251</td>
<td>0.024</td>
</tr>
<tr>
<td>MDA</td>
<td>-</td>
<td>-</td>
<td>-0.372</td>
</tr>
<tr>
<td>HDL-C</td>
<td>-0.093</td>
<td>ns</td>
<td>-0.177</td>
</tr>
</tbody>
</table>
dual role, with regard to the complex mechanism of tumour invasion and metastasis and could either mediate tumoricidal activity or promote tumour growth\textsuperscript{34}. Its presence has been found in various human malignant tumours, including breast, renal, pancreas, ovarian and lung\textsuperscript{\textsuperscript{17,35-38}}. Some workers have reported a higher NO synthase activity in tumours\textsuperscript{\textsuperscript{17,36}}, while some have reported a lower activity\textsuperscript{\textsuperscript{37,38}}. In the present study, serum nitrite levels were found elevated in breast cancer patients and were highest in stage IV (approx. twice than that in stage III). Increased nitrite levels could be due to the tumour-inducing NO synthase activity.

ROS-induced lipid peroxidation has been implicated in neoplastic transformation. The lipid peroxidation products such as MDA (used as an indicator of lipid peroxidation) and hexanal can structurally alter DNA, proteins and other biomolecules. Increased lipid peroxidation in serum and tissues has been reported in breast cancer patients\textsuperscript{\textsuperscript{16,18,28,39,40}}. In our study, serum MDA levels were elevated in the total patients and clinical stage groups (stage II, III, IV); the levels being highest in stage IV. A positive correlation was found between MDA and nitrite levels, in the total group.

Vit. E as a lipophilic antioxidant molecule is able to react with lipid peroxyl radicals, eventually terminating the peroxidation chain reaction, and thereby reducing oxidative damage\textsuperscript{41}. It induces cancer cells to undergo apoptosis\textsuperscript{42}. Previous studies reported inconsistent results on vit. E in breast cancer patients\textsuperscript{\textsuperscript{19-21}}. In this study, vit. E levels were significantly decreased, in particular, in stage II and IV patients, as compared to controls. This may be due to its increased utilization to scavenge lipid peroxyl radicals in tumour cells. We found a negative correlation between vit. E and nitrite levels and between vit. E and MDA levels. Vit. E is reported to significantly improve the resistance of pancreatic cells to toxic doses of nitrite\textsuperscript{43}. Also, its administration reduce the concentrations of lipid peroxides\textsuperscript{44}.

Lipids and lipoproteins have also been associated with the breast cancer\textsuperscript{45}. Low HDL-C is associated with increased postmenopausal breast cancer risk\textsuperscript{46}. Previous studies reported decreased serum HDL-C levels with higher TG levels in cancer\textsuperscript{\textsuperscript{19,45,47}}. On the contrary, higher serum HDL-C levels have also been reported in breast cancer patients\textsuperscript{48}. In our study, when compared with healthy controls, serum TG levels were increased in total and stage IV patients, however, HDL-C levels were diminished in total and stage II, III, IV patients. Vit. E circulates in blood with the LDL-C fraction, however, no correlation was found between these parameters. A positive correlation was found between HDL-C and vit. E in the total group, which was in agreement with an earlier report\textsuperscript{19}. As vit. E and HDL-C levels were found decreased in breast cancer patients, they may not be sufficient enough to counter free radical attack, thereby resulting in oxidative stress.

In conclusion, the study demonstrated higher serum MDA, nitrite and TG levels and significantly decreased levels of vit. E and HDL-C among breast cancer patients. An association between oxidative stress and antioxidant protection was observed in the present study. However, studies with more patients and oxidative stress-related parameters are required, to explore the relationship between free radicals and antioxidants, in relation to promotion and malignant conversion stages of breast cancer.

Acknowledgement
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