Serum and RBCs Glutathione Reductase (GSSG-Red)
Activity in Breast Tumors of Iraqi Women

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Abstract
Breast tumor patients generally have more oxidative stress than normal females. This was clear from non significant (P>0.05) decrease in RBCs GSSG-Red but there was significant decrease (P<0.05) in serum GSSG-Red activity. The study had found that free radicals in malignant breast tumors were higher than benign tumors, therefore the activity of GSSG-Red might be used as markers for prognosis of the disease through previous studies and present study.

Introduction
The breast is a large compound racemose gland, consists of a few ducts, which are connected to the nipple and open to the surface. The main function of the breast is the production and expression of milk. Development of the breast requires the co-ordinate action of many hormones(1).

The breast tumors might be described, as either benign or malignant (cancerous), and the breast cancer is the most common in many women in developing countries. The national center of cancer in Iraq found that there was an increased incidence of breast cancer in Iraqi women for years (1975-2000)(2).

Reactive oxygen species (ROSs) are the main cause of breast cancer which encompasses all highly reactive oxygen containing molecules, including free radicals(3). Some of ROSs are defined as a free radicals, any atom or molecule having an unpaired electron in its outer orbit as (O$_2^-$, OH*, COO*, CO$^*_{3}$). Others, are not radicals but active metabolites of
oxygen, e.g. (H$_2$O$_2$, HOCl). ROSs formation sources are mitochondrial respiratory chain, phagocytes, redox reaction, radiation, cigarette, smoke environmental pollution. Main ROSs are: (super oxide radical O$_2^{-}$, Hydrogen peroxide H$_2$O$_2$, hydroxyl radical OH$^*$, nitric oxide NO, singlet oxygen O$_2^*$). A first line body defense against oxidative stress produced by generation of free radical and reactive oxygen species ROSs are antioxidants which can defined as (any substance which delays or inhibits oxidative damage to a target molecule$^{(4)}$).

Antioxidants are classified to enzymatic (glutathione peroxidase, glutathione reductase, super oxide dismutase, catalyase...) and non enzymatic (vitamineC, vitamineE, bilirubin,glutathione$^{(5)}$).

GSSG- Red (EC 1.6.4.2) molecular mass for the native enzyme (110 KD) and for each subunit of the dimer (55KD)$^{(6)}$. The homodimeric enzyme is a member of the family of flavoprotein disulfide oxidoreductase. Each subunit has four domains; beginning at the N- terminus: an FAD-binding domain; an NADPH- binding domain, a central domain and an interface domain$^{(6)}$. The active site of GSSG- Red is at the dimeric interface, also it carries a redox active disulfide (Cys- 58- Cys- 63) in its active site which is reduced by electron transfer from NADPH via the flavin$^{(7,8)}$.

Since the GSSG binding site is composed of residues form both subunits, only the dimeric form is active$^{(9,10)}$.

$$GSSG + NADPH + H^+ \xrightarrow{GSSG-Red} 2GSH + NADP^+$$

Oxidized glutathione is reduced by a multi- steps reaction as shown below$^{(11,12)}$: -
The main inhibitors of GSSG-Red (unknown mechanism): Oxidized glutathione GSSG\(^{(13)}\), NADPH\(^{(14)}\), and \(p\)-hydroxy mercuribenzoic acid\(^{(15)}\). Another studies suggested some inhibitors of GSSG- Red and the mechanisms of inhibition as: Becker et al., found two ways to inhibit crystalline erythrocyte GSSG- Red:

(a) As a reversible inhibitor:

Inhibitor is competitive with glutathione disulfide (GSSG), the \((K_i)\) being approximately 0.5mM.

(b) As an irreversible inhibitor:

This inhibitor depends on the presence of NADPH and could not be reversed by dilution nor by reducing agents\(^{(16)}\). Whereas Petrickova et al., suggested that the inhibition effect of tested polyanions was caused by electrostatic interactions with enzyme. The kinetic analysis indicated that it is a mixed inhibition with respect to oxidized glutathione of NADPH\(^{(17)}\). Some inhibitors of this enzyme are: (ZnCl\(_2\), nitrosative stress, inorganic; organic arsenic, dextran sulfate & heparine)\(^{(18,19,21,22)}\).

An investigation by Coban et al., for GSSG-Red activity in normal & neoplastic human breast tissue, found that the mean activities of GSSG-Red in tumour tissues were significantly higher than those in normal tissues\(^{(20,23)}\), as well as Seven et al., studied the oxidative stress & GSSG-Red activity by measuring GSH redox cycle parameters in benign & malignant breast disease\(^{(24)}\).

**Materials & Methods**

**Subjects:** The study included 56 female subjects who were admitted for diagnosis & surgery to (Al- Yarmok Teaching Hospital, Baghdad Teaching Hospital, Nursing Home Hospital). Patients suffered from any diseases that may interfere with this study were excluded.

<table>
<thead>
<tr>
<th>Group</th>
<th>Patients</th>
<th>No.</th>
<th>Age</th>
<th>Type of tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>pre-menopausal benign breast tumors</td>
<td>15</td>
<td>16-49</td>
<td>- Fibrocystic changed adenosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- Axillary lymph nodes</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- Fibro adenoma</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- Duct papilloma</td>
</tr>
<tr>
<td>II</td>
<td>pre-menopausal malignant breast tumors</td>
<td>4</td>
<td>16-49</td>
<td>- Infiltrative ductal carcinoma</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- InSitu carcinoma</td>
</tr>
<tr>
<td>III</td>
<td>postmenopausal benign breast tumors</td>
<td>5</td>
<td>50-65</td>
<td>- Duct Papilloma</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- Axillary Lymph nodes</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- Fibrocystic Changed</td>
</tr>
<tr>
<td>IV</td>
<td>postmenopausal malignant breast tumors</td>
<td>6</td>
<td></td>
<td>- Infiltrative ductal carcinoma.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- Fat necrosis.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Several patient’s with metastasis &amp; recurrence breast carcinoma after mastectomy &amp; radio or chemotherapy.</td>
</tr>
<tr>
<td>V</td>
<td>Control</td>
<td>26</td>
<td>20-45</td>
<td></td>
</tr>
</tbody>
</table>

Non of the patients were on a special diet, or taking any antioxidants (Vitamins E, Vitamins C, … etc) or treated with antioxidant drugs except
(Voltarin, Ampy Glucose, Paracetol), non of patients were exposed directly to radiation and didn’t drink alcohol or smoke, negative genetic factor to have cancer with very clearly irregular menstrual cycle in most patients.

**Collection of blood samples:**
Venous blood samples collected from each subject just before surgery using a (5 ml) steril syringe and needle. Tow & half milliliter (2.5ml) of aspirated blood was immediately transferred into plein tube with anticoagulant (Potassium-EDTA) (Ethylene diamine tetra acetate tripotassium) for erythrocytes isolation, then centrifuged at 4000/ r.p.m for 10 minutes. Plasma & buffy coat were removed and erythrocytes were washed two times with cold normal saline (0.9% NaCl) pH= 7.0. The packed cell volume (PCV) stored at (-20°C) until assay. Another (2.5ml) of aspirated blood were transferred into plein tube without anticoagulant for serum separated by centrifugation at 4000 r.p.m for 10 minutes (serum kept at -20°C until assay).

**Determination of GSSG_Red activity:**
The enzymatic assay of GSSG_Red activity was assayed by Lee method (1975) using oxidized glutathione (GSSG) as substrate and NADPH as coenzyme. The GSSG_Red activity was expressed in term of (U/L) in serum and (U/g Hb) in RBCs(25).

**Statiscal method:**
The data obtained are presented as mean, SD, and significance of the differences between both groups was assessed by student's t_test. P<0.05 was considered significant.

**Results and Discussion**
Table (2) shows that in RBCs there was a non-significant decrease in enzyme activities for malignant post M.P. patients as compared to that found in normal subjects, but in malignant pre M.P patients there was a significant increase (P<0.05) compared to normal controls.

It was noticed that there was no change in the activity in patients with benign tumors post M.P, while in pre M.P, had a non-significant increase in GSSG-Red activity.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>NO.</th>
<th>Mean ±SD</th>
<th>Probability</th>
<th>P.value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>34</td>
<td>0.750 ±0.281</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malignant PreM.P.</td>
<td>5</td>
<td>0.789 ±0.148</td>
<td>0.038</td>
<td>3.044</td>
</tr>
<tr>
<td>Malignant PostM.P.</td>
<td>6</td>
<td>0.639 ±0.075</td>
<td>0.210</td>
<td>1.437</td>
</tr>
<tr>
<td>Benign PreM.P.</td>
<td>15</td>
<td>0.820 ±0.264</td>
<td>0.357</td>
<td>0.951</td>
</tr>
<tr>
<td>Benign PostM.P.</td>
<td>4</td>
<td>0.753 ±0.237</td>
<td>0.982</td>
<td>0.025</td>
</tr>
</tbody>
</table>

*GSSG-Red activity :The amount of enzyme which catalyze convert 1µmol of GSSG as substrate to GSH as product during 5 minutes in 1 gm of Hb under pH=7.4 T=25°C .
Red blood cells (RBCs) protect their cellular integrity and biovitel molecules such as proteins, enzyme & membrane lipids from the injurious effects by ROS, primarily $H_2O_2$ & by conserving a constant high ratio of GSH/GSSG$^6$. The enzymatic activity that responsible for maintaining high intracellular GSH to GSSG is achieved by glutathione reductase. The enzyme GSSG-Red can catalyze the conversion of oxidized glutathione GSSG to the corresponding reduced form GSH & the reaction proceeds by the presence of NADPH which is converted to NADP$^+$.

\[
\text{GSSG} \xrightarrow{\text{GSSG-Red}} 2 \text{GSH} + \text{NADP}^+ \rightarrow \text{NADPH} + \text{H}^+ 
\]

The enzymatic activity can be monitoring by measuring the decrease in the absorbance at 340 nm$^{25}$. In malignant & benign tumors the activity of GSSG-Red in pre M.P. was more than post M.P patients. In general, patients with benign tumors had GSSG-Red activities more than those in malignant for both pre & post M.P.

Decreased GSSG-Red activity in RBCs, was in disagreement with another studies in leukemia$^{26}$, and this in disagreement with study of Sabitha & Shaymaladevi$^{27}$, who found significant decrease in the activities of red blood cell hemolysate antioxidant enzymes such as glutathione peroxidase and glutathione reductase in oral cancer patients which represent the lack of antioxidant defense.

As shown in table (3) serum GSSG- Red activities in patients with malignant & benign tumors for both pre & post M.P women was significantly decreased (P<0.05) compared with normal subjects.

<table>
<thead>
<tr>
<th>GROUP</th>
<th>NO.</th>
<th>Mean</th>
<th>±SD</th>
<th>Probability</th>
<th>P.valu</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>26</td>
<td>7.050</td>
<td>0.928</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malignant</td>
<td>PreM.P.</td>
<td>4</td>
<td>4.572</td>
<td>0.518</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>PostM.P.</td>
<td>6</td>
<td>4.292</td>
<td>1.417</td>
<td>0.008</td>
</tr>
<tr>
<td>Begnin</td>
<td>PreM.P.</td>
<td>15</td>
<td>5.293</td>
<td>1.760</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>PostM.P.</td>
<td>5</td>
<td>5.051</td>
<td>0.698</td>
<td>0.011</td>
</tr>
</tbody>
</table>

*GSSG-Red activity :The amount of enzyme which catalyze convert $1 \mu$mol of GSSG as substrate to GSH as product during 5 minutes in 1 Liter of serum under pH=7.4 T=25ºC.
In malignant & benign tumors serum GSSG-Red activity in Pre M.P was more than in post M.P patients.

Generally, benign tumors’ patients had enzyme activity more than those in malignant for both pre & post M.P.

Our results were in an agreement with other studies in plasma, leukocyte, lymphocyte of acute leukemia\(^{28}\), and oral cancer\(^{29}\).

Salim et al., observed also a significant inhibition in serum GSSG-Red activity of patients with leukemia\(^{30}\).

References


